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Bacteriovorax stolpii proliferation and predation without sphingophosphonolipids

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Abstract

Bacteriovorax stolpii strain UKi2, a facultative predator-parasite of larger Gram-negative bacteria, synthesizes distinct sphingophosphonolipids. These lipids are characterized by a direct P–C bond, the novel head group 1-hydroxy-2-aminoethylphosphonate, iso-branched long chain bases and fatty acids, and fatty acids dominated by those with α-hydroxy groups. Myriocin, an inhibitor of serine:fatty acyl CoA transferase, reversibly blocked sphingophosphonolipid synthesis in *B. stolpii* UKi2. However, the inhibitor did not block cell proliferation indicating that these lipids are not vital for *B. stolpii* UKi2 viability and growth. When mixed with *Escherichia coli* prey cells, control predator-parasite bacteria were effective in forming large *E. coli* bdelloplasts and cleared the suspension of the prey cells. Although myriocin-treated cells could attack prey cells and form bdelloplasts, their locomotory behavior was altered and fewer and smaller bdelloplasts were produced. These observations open up the possibility for a role of sphingophosphonolipids in *B. stolpii* UKi2 complex behavior.

Keywords: Bdelloplasts; Escherichia coli; Long chain base; Myriocin; Parasite; Predator; Sphingolipids; Serine:fatty acyl CoA transferase

Bdellovibrios-and-like organisms (BALO) are small comma- or crescent-shaped, flagellated predatory bacteria that swim rapidly attacking larger Gram-negative bacteria [1–3]. After penetrating the prey's periplasm the bacterium loses its flagellum and begins its parasitic phase. The infected host cell containing the parasite, which proliferates as a filament within the cytoplasm is called a bdelloplast. Differentiated individual progeny cells are released during lysis of the host cell. Because of their predatory and parasitic life style, these bacteria are being considered as potential agents in treating environmental pollution, purifying drinking water systems, disrupting biofilms, and clearing bacterial infections [2,3].

Sphingolipids are commonly found in eukaryotic cell membranes [4–15] and have been best studied in the ciliated protozoa *Tetrahymena* and *Paramecium* [4–8], yeast [9–11] and in various mammalian species [14,15]. On the other hand, sphingolipids are rarely found in bacteria and have thus far only been reported in the genera *Sphingomonas* [16], *Sphingobacterium* [17–20] *Bacteroides* [21], *Flavobacterium* ([22], and *Bacteriovorax stolpii* UKi2 [20,23–25]. *Bacteriovorax stolpii* (formerly, *Bdellovibrio bacteriovorus*, *Bdellovibrio stolpii*) [23,26,27] strain UKi2 contains at least 18 molecular species of rare sphingophosphonolipids (SPNL) [23–25]. *B. stolpii* UKi2 is the only bacterium known to contain these lipids. The structures of the major *B. stolpii* UKi2 SPNL were initially elucidated over 30 years ago by their chemical properties and by thin-layer and gas–liquid chromatographic techniques [23]. Since 2001, information on the definitive structures and individual molecular species identified by state of art mass spectrometry and nuclear magnetic resonance spectroscopy became available [24,25]. The dominant SPNL molecular species are characterized by a direct P–C bond, an unusual

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head group 1-hydroxy-2-aminoethylphosphonate, long chain bases and fatty acids with iso structures, and fatty acids with α-hydroxyl groups. Recently, Ikushiro et al. examined serine:fatty acyl CoA transferases in *Sphingomonas*, *Sphingobacterium* and *B. stolpii* UKi2 using recombinant protein and immunofluorescence approaches [16,20]. The *B. stolpii* UKi2 and *Sphingobacterium* enzymes apparently were found localized in the inner membrane of the cells unlike that of *Sphingomonas* where it is in the cytosol [16]. Unlike the other bacterial serine:fatty acyl CoA transferases, the recombinant *B. stolpii* UKi2 enzyme differed with respect to enzyme kinetics in that it was strongly inhibited by >100 μM palmitoyl CoA [20].

In 1973, Steiner et al. reported that *B. stolpii* strain UKi1 a free-living, host-independent strain derived from the same obligate host-dependent parental strain that gave rise to strain UKi2 lacked both predatory behavior and SPNL [23]. Thus, their report strongly suggested a role of these lipids in the predatory behavior of *B. stolpii* and/or its ability to live as a parasite within other bacteria. In the present study, we produced UKi2 cells lacking SPNL by employing an inhibitor of sphingolipid synthesis then examined the consequences of this treatment.

Materials and methods

Bacteriovorus stolpii UKi2 cultures. Bacteriovorax stolpii UKi2 (ATCC 27052) was grown axenically as standing cultures in 0.3% yeast extract [24] at 30 °C. Culture density was monitored by Abs_{660nm}. Alternatively, after demonstrating a direct relationship between cellular ATP levels and bacterial numbers, quantitation of ATP was used to monitor cell density (Lumitester K-210, Kikkoman Corp., Noda, Japan).

Myriocin (2*S*,3*R*,4*R*,6*E*-2-amino-3,4-dihydroxy-2-hydroxymethyl-14-oxo-6-eicosenoic acid; ISP-1; thermozymocidin) (BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) [28] inhibits serine:fatty acyl CoA transferase, the first committed step in sphingolipid synthesis that produces long chain bases (LCB). In eukaryotes, myriocin blocks this reaction thus inhibiting sphingolipid synthesis.

Myriocin dissolved in 2% dimethylsulfoxide (DMSO) was added at various concentrations to the culture medium and dispersed by sonication. The final DMSO concentration in bacterial cultures was <1%, which did not inhibit *B. stolpii* cell proliferation. To test whether myriocin had an effect on the incorporation of radiolabeled serine into *B. stolpii* lipids, control cultures were grown with 10 μCi L-[U–^{14}C]serine (Amersham, UK) for 24 h at 30 °C. Experimental cultures were incubated with 10 μCi L-[U–^{14}C]serine plus 50 μM or 100 μM myriocin.

To examine whether the effects of myriocin treatment on SPNL syntheses was reversible, *B. stolpii* UKi2 cultures were first grown overnight with 75 μM myriocin. The cells were then harvested and washed three times by centrifugation and resuspension in fresh 0.3% yeast extract containing 1% DMSO then once with 0.3% yeast extract alone. After 24 h cultivation in 0.3% yeast extract they were subcultured and allowed to grow for another 24 h. Cells that were under myriocin treatment and the first and second subcultures in drug-free medium were harvested and lipids were extracted and analyzed as described below.

Lipid analysis. Total lipids were extracted in $CHCl_3$:CH_3OH (2:1, v/v) and lipid classes were separated by 1D HPTLC [25] using the solvent system *n*-propanol:*n*-propylamine:H_2O (80:15:5, v/v/v; SS#1) then the plates were stained for phosphorus [29] or visualized by iodine vapor. Nonradioactive or metabolically radiolabeled lipids were also separated by 2D HPTLC by development in $CHCl_3$:MeOH:H_2O (60:35:8, v/v/v), and after the solvent front reached the top, it was dried and redeveloped in the same direction using $CHCl_3$:MeOH:acetic acid (90:2:8, v/v/v). The plate was developed in the second dimension using SS#1. The 2D HPTLC plates containing radiolabeled lipids were subjected to radioautography (Fuji medical X-ray film RU–X, Fuji Film, Japan).

Co-incubation of predator and prey. Prey *Escherichia coli* (ATCC 11303) was grown overnight at 37 °C in a shaker incubator at 300 rpm (Beckman, Fullerton, CA) in l0.8% nutrient broth plus 0.5% yeast extract (Difco Laboratories); pH 7.6. After twice washing *E. coli* and *B. stolpii* UKi2 cultures in a buffered solution (10 mM HEPES, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, pH 7.6) the cells were resuspended in this non-nutrient buffer solution and equal volumes of each were combined. The mixed cell suspension was incubated by shaking at 250 rpm for 15 min at 30 °C during which time bdelloplasts form [1]. In some experiments, *B. stolpii* cultures were grown for 48 h with 50 μM myriocin to inhibit SPNL synthesis. After washing by centrifugation and resuspension in the buffered solution, the cells were mixed with prey cells. Microscopic observations of the organism mixtures were performed on a Nikon Optiphot microscope using differential interference optics. Micrographs were obtained by a Spot II camera (Diagnostics Instruments, Inc., Sterling Heights, MI) at 1000× magnification.

Results and discussion

Bacteriovorus stolpii UKi2 axenic cultures and effects of myriocin

Cultures of *B. stolpii* UKi2 were characterized and late-log to early-stationary phase was found to occur at approximately 24 h (Fig. 1A). Under these conditions, myriocin at various concentrations up to 100 μM had no inhibitory effect on *B. stolpii* UKi2 culture growth compared to DMSO controls after 24 h incubation (Fig. 1B).

Lipid analyses

The phospholipid profile of cells visualized after separation by 1D HPTLC resolved the major glycerophospholipid and sphingophosphonolipid classes present in this bacterial strain (Fig. 2, lane 1). These analyses confirmed the report by Steiner et al. [23] that the dominant glycerophospholipids in *B. stolpi* UKi2 were PE and PG and that PS was a minor component. The identity of PG and PS was verified by mass spectrometry (L. Sallans, Y. Watanabe, E.S. Kaneshiro, unpublished).

Effects of myriocin

The lipid profile of *B. stolpii* UKi2 grown with 0.99% DMSO, the primary solvent used for dissolving myriocin (Fig. 2, lane 2), was similar to that of controls. In contrast, the slower migrating sphingophosphonolipids were absent in cells grown with 0.99% DMSO plus 50 μM myriocin (Fig. 2, lane 3); similar results were observed with 100 μM myriocin. Thus, the compound inhibited *B. stolpii* sphingolipid synthesis as it does in eukaryotes.

Additional evidence for the mechanism of myriocin inhibition of SPNL synthesis was provided by the results of metabolic radiolabeling experiments indicating that *B. stolpii* UKi2 SPNL LCB synthesis involves the same initial condensation reaction that utilizes serine. Autoradiograms of *B. stolpii* UKi2 lipids metabolically radiolabeled with

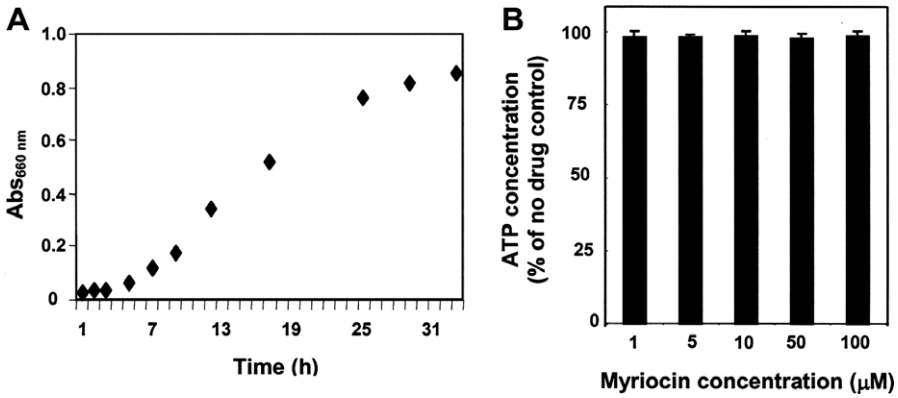

Fig. 1. (A) Culture growth of *B. stolpii* UKi2 in 0.3% yeast extract. Late-log to early-stationary phase is achieved at approximately 24 h. (B) *B. stolpii* UKi2 cultures grown with various concentrations of myriocin (bar = SD; $n = 5$). The drug did not inhibit cell proliferation up to 100 μM of the drug. Culture proliferation was monitored by measuring cellular ATP after establishing a strict correlation with cell number and ATP.

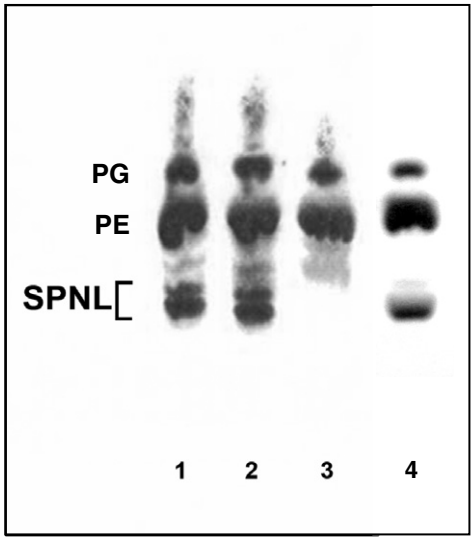

Fig. 2. 1D HPTLC separation of *B. stolpii* total lipids developed in CHClthen stained for phosphorus. Lane 1, untreated control cells; SPNL, sphingophosphonolipids. Lane 2, cells grown with the primary solvent 0.99% DMSO. These cells contain SPNL as found in control cells. Lane 3 cells grown with 50 μM myriocin plus 0.99% DMSO. The inhibitor totally blocked the synthesis of the SPNL. Lane 4, cells in the second subculture after myriocin treatment. These cells had the ability to synthesize SPNL indicating the effect of the drug on SPNL synthesis was reversible.

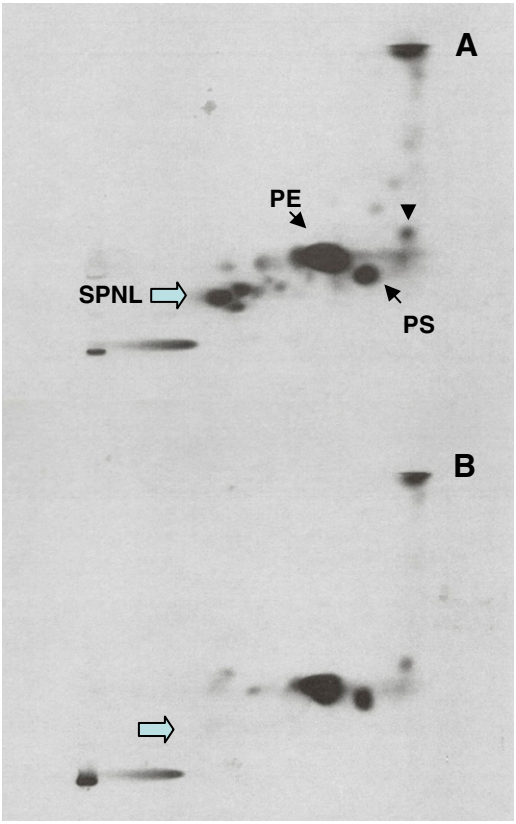

Fig. 3. Autoradiograms of 2D HPLC separations of *B. stolpii* UKi2 lipids when cells were grown with [U–^{14}C]serine. (A) Lipids of untreated control cells. Radiolabeled serine was incorporated into the SPNL components (arrow). The major glycerophospholipids PE and PS were also radioactive. (B) Cells grown under the same conditions but in the presence of 50 μM myriocin, radiolabeled serine was incorporated into the glycerophospholipids but not the SPNL, indicating SPNL synthesis was specifically inhibited.

[U–^{14}C]serine and separated by 2D HPTLC indicated that the SPNL and the glycerophospholipids PE and PS were radioactive (Fig. 3A).

We conclude that radiolabeled serine was readily incorporated into PE and PS (Fig. 4), which is consistent with the production of PE by decarboxylation of serine in PS [8] and suggests that *B. stolpii* UKi2 has the enzymes that can convert glycerophospholipids by action at the polar head group. The distinct spot above PS that also incorporated radioactivity from serine is tentatively identified by *N*-acetylphosphatidylethanolamine (arrowhead in Fig. 3A). This glycerophospholipid was identified in *Bdellovibrio bacteriovorus* (N.-A. Nguyen, L. Sallans, E.S. Kaneshiro, unpublished data) and its migration in this 2D HPTLC system is the same as this *B. stolpii* UKi2 radioactive lipid component. Synthesis of *N*-acetylphosphatidylethanolamine could result from head group acetylation of PE, which would be consistent with its being radioactive.

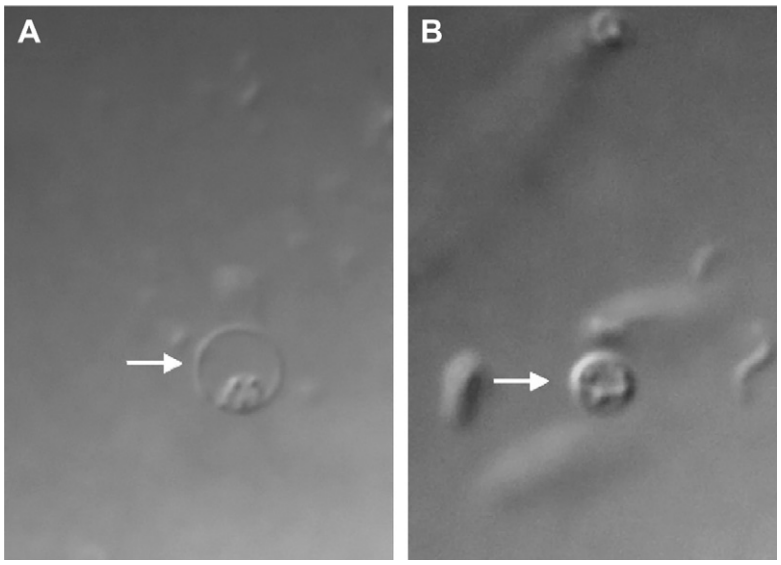

Fig. 4. Microscopic observations of the effects of myriocin on the ability of *B. stolpii* to form *E. coli* bdelloplasts. (A) Untreated control cells were readily capable of attacking *E. coli* forming large bdelloplasts resulting in the lysis of the host cells and clearing *E. coli* from the mixed cell suspension. (B) Cells grown with 50 μM myriocin were capable of predation and formed *E. coli* bdelloplasts. However, fewer and smaller bdelloplasts were detectable in the mixed cell suspension.

Autoradiograms of 2D HPTLC separations of lipids from myriocin-treated cells grown with radiolabeled serine demonstrated that these cells did not contain radioactive SPNL (Fig. 3B). These results are consistent with the conclusion that myriocin was effective in blocking the condensation of serine with a fatty acyl CoA in *B. stolpii* UKi2. Together, the experiments on culture proliferation, cellular phospholipids, and radiolabeled serine incorporation demonstrated that SPNL are not vital for viability and growth of *B. stolpii* UKi2 cells in a nutrient culture medium.

The inhibition of SPNL synthesis by myriocin was reversible. After growing cells in 75 μM myriocin then washing the cells, growth in two subcultures were required to eliminate the relatively water-insoluble compound from the cells and from the biosynthetic machinery in them. Nonetheless, UKi2 cells in the second subculture were fully capable of synthesizing SPNL (Fig. 2, lane 4). Hence myriocin-treated cells were not permanently altered in their ability to synthesize these lipids as had apparently occurred by mutation that produced strain UKi1.

Co-incubation of predator and prey

Bacteriovorax stolpii UKi2 cells were effective predators of *E. coli* as indicated by the presence of numerous large bdelloplasts (Fig. 4A) when washed prey and predator cells were mixed. The *E. coli* cells were eventually cleared from the mixed cell suspension. These observations confirm that this strain should not be described as strictly host-dependent or host-independent but indeed is accurately designated as a facultative predator and parasite.

Myriocin-treated *B. stolpii* were also capable in forming *E. coli* bdelloplasts. However, under the same incubation conditions, only a few (<50%) bdelloplasts were formed compared to untreated controls (Fig. 4B). This suggested that the ability of *B. stolpii* UKi2 to enter prey cells was altered and intracellular growth of the cells that managed to penetrate *E. coli* was also affected. Importantly, it was noted that myriocin-treated *B. stolpii* UKi2 cells swam slower and exhibited a tumbling type of locomotion. Locomotory behavior, predation, penetration into prey cells, and growth within the prey cell are complex processes and while some progress in understanding parts of these events have been accomplished [30–36], much remain unknown. It is currently not understood what role SPNL play in *B. stolpii* UKi2 but these lipids do not appear to be required for predation and parasitism per se. On the other hand, if the biochemical composition of the cell's flagellar sheath is altered by the lack of these lipids, it might be expected that the cell's locomotory behavior would be affected and changes in the effectiveness in predation and parasitism would be probable consequences. There is currently a lack of information on the lipids of a broad representation of bdellovibrios. Hence there is a need to examine the distribution of these important compounds among more species and strains of these predatory-parasitic bacteria.

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