Ability of *Bdellovibrio bacteriovorus* 109J to Lyse Gram-Negative Food-Borne Pathogenic and Spoilage Bacteria

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ABSTRACT

Bdellovibrios are a group of aerobic, predatory bacteria which attack, penetrate and grow in many species of gram-negative bacteria, causing the lysis of the invaded prey organism. *Bdellovibrio bacteriovorus* strain 109J varied in its ability to lyse 32 bacterial strains comprising six genera of food-borne pathogens and spoilage organisms. The reduction in the levels of the prey bacteria ranged from 0.1 to 7.7 log-values after 7 h of incubation at 30°C. *Escherichia coli* strain 2239-69 (pathogenic serotype 026:H11) was lysed most effectively at temperatures between 30 and 37°C, however, lysis also occurred at 12 and 19°C when the incubation period was extended to 24 h. *Bdellovibrio bacteriovorus* was effective in reducing the level of *E. coli* 2239-69 at pH 5.6 to 8.6. Increasing the *Bdellovibrio: E. coli* ratio resulted in a more rapid *E. coli* reduction. This study demonstrated the potential usefulness of bdellovibrios for the biological control of pathogenic and spoilage organisms in foods.

Key words: *Bdellovibrios bacteriovorus* 109J, lyse gram-negative, *Escherichia coli*

Microbial food safety is an issue of major concern to the food industry, governmental regulatory agencies and to the consumer, therefore, the development of strategies directed toward the elimination or control of food-borne pathogens is essential. Techniques under investigation aimed at controlling the level of pathogens in foods include the use of low-dose irradiation (*13*) or of bacteriocins (*6*), organic acids and other chemical agents (*1*). The use, in foods or on agronomic crop plants, of microorganisms which are parasitic on other bacteria has only been explored to a limited degree (*4,8*).

Bdellovibrios are unique predatory microorganisms capable of attaching to suitable prey bacteria, penetrating the cell wall and multiplying in the periplasmic space while digesting the prey cell contents (*10,12*). The prey bacterium eventually lyses, releasing progeny bdellovibrios, which initiate a new life cycle in other suitable prey organisms. The present study is the first to systematically examine the ability of *B. bacteriovorus* 109J to prey upon a variety of gram-negative food-associated pathogenic and spoilage organisms. The effects of pH of the medium, incubation temperature and predator:prey ratio were also investigated using *E. coli* 2239-69 as the prey strain.

MATERIALS AND METHODS

Bdellovibrio and prey strains

Bdellovibrio bacteriovorus 109J, ATCC 43826, was provided by John Tudor, St. Joseph's University, Philadelphia, PA. The bacterial strains used as substrate cells and their sources are listed in Table 1.

Maintenance of Bdellovibrio bacteriovorus

Bdellovibrio bacteriovorus was propagated weekly on *E. coli* ML35, ATCC 43827, by adding 2 ml of the lysate (progeny bdellovibrios and lysed substrate bacteria) to 8 ml of substrate bacteria (*E. coli*) in dilute nutrient broth (DNB) (*9*) consisting of 0.08% nutrient broth (Difco Laboratories, Inc., Detroit, MI), 0.05% casamino acids (Difco), 0.01% yeast extract (Difco), 2mM $CaCl_2$ x $2H_2O$ (5 ml of 0.2M stock added to 500 ml cooled medium) 3mM $MgCl_2$ x $6H_2O$, pH 7.4, and incubating at 30°C for 16 h at 250 rpm. The lysates containing a fresh crop of *Bdellovibrio* were kept at 4°C.

Cultivation of Bdellovibrio *and prey bacteria*

To cultivate *B. bacteriovorus* to concentrations required for the two-membered culture experiments, the organism was propagated as described by Jackson and Whiting (*4*). Two milliliters of *Bdellovibrio lysate* was added to 200 ml of *E. coli* ML35 in DNB. The predator:prey suspension was incubated aerobically for 18 h at 30°C at 250 rpm. Following centrifugation, the pellet, containing *Bdellovibrio*, was suspended in HEPES metal buffer (HMB) (*4*), pH 7.4 or at other specified pH values. The prey bacteria were grown for 18 h in nutrient broth at 37°C with aeration, and following centrifugation, were resuspended in HMB.

**Reference of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.*

TABLE 1. *Measurement of effectiveness of* B. bacteriovorus *109J at reducing the level of gram-negative substrate bacteria.*

Substrate bacteria	Source[a]	Log reduction in prey population after 7h[b,c]	Control[b]
Aeromonas hydrophila K144	UM	4.24	0.19
Aeromonas pappu	ULS	5.05	0.20
Escherichia coli (O157:NM)	ECRL	0.37	-0.02
Escherichia coli 3417-85 (O157:H7)	CDC	0.80	0.33
Escherichia coli A9124-1 (O157:H7)	CDC	0.60	-0.05
Escherichia coli 45753-35 (O157:H7)	FSIS	0.36	0.23
Escherichia coli 88-1558 (O157:H7)	ECRL	0.51	-0.30
Escherichia coli 11602 (O159:H34)	CPHL	7.68	0.24
Escherichia coli 1801-72 (O26:H11)	CDC	5.12	0.36
Escherichia coli 2239-69 (O26:H11)	CDC	5.54	-0.13
Escherichia coli 3359-70 (O26:H11)	CDC	5.54	-0.07
Pseudomonas aeruginosa DAR 41352	ERRC	5.20	-0.20
Pseudomonas putida 17392	ATCC	5.75	0.33
Pseudomonas fluorescens 17816	ATCC	7.06	-0.22
Salmonella arizonae 29933	ATCC	0.50	-0.19
Salmonella dublin 15480	ATCC	1.15	0.13
Salmonella enteritidis 92-008	UPVS	0.10	-0.27
Salmonella enteritidis 5-19	UPVS	0.83	0.41
Salomonella enteritidis Y8P2	UPVS	0.77	0.20
Salmonella enteritidis 2000	ERRC	0.77	0.33
Salmonella seftenberg Pro 168	UPVS	0.69	0.12
Salmonella poona	AMS	6.43	-0.22
Salmonella typhimurium TML R66	ERRC	0.72	0.37
Salmonella typhimurium 14028	ATCC	0.48	0.00
Shigella flexneri 5348	UTMB	6.61	-0.47
Shigella flexneri 20029	FDA	7.47	-0.11
Shigella sonnei 20014	FDA	7.18	-0.28
Shigella dysenteriae 20011	FDA	4.70	-0.01
Yersinia enterocolitica O:Tacoma	FDA	2.70	0.05
Yersinia enterocolitica GER O:3	FDA	7.69	0.29
Yersinia enterocolitica WA O:8	FDA	2.70	0.23
Yersinia enterocolitica PT18 O:5, 27	FDA	2.94	0.12

[a]UM, University of Maryland, College Park, MD; ULS, University of Lund Solvegatan, Lund, Sweden; ECRL, *E. coli* Reference Laboratory, University Park, PA; CDC, Centers for Disease Control; FSIS Food Safety and Inspection Service; CPHL, Central Public Health Laboratories, London, England; ERRC, Eastern Regional Research Center, Philadelphia, PA; ATCC, American Type Culture Collection; UPVS, University of Pennsylvania Veterinary School; AMS, Agricultural Marketing Service, Gastonia, NC; UTMB, University of Texas Medical Branch, Galveston, TX; FDA, Food and Drug Administration.

[b]Log value of number of prey bacteria at time 0 h minus log value of number of prey bacteria at time 7 h.

[c]The difference in the log values of the number of surviving prey bacteria of the replicate experiments was within 0.5 log units.

Determination of prey organism range for B. bacteriovorus *109J (two-membered cultures)*

The *Bdellovibrio* and prey cell suspensions were mixed in 50 ml Erlenmeyer flasks at a ratio of 2:1 (predator:prey) in a total volume of 10 ml of HMB with cell concentrations at 5 x 10^9 colony forming units (CFU)/ml of prey and 1 x 10^{10} plaque-forming units (PFU)/ml of *Bdellovibrio*. The method utilized for PFU determination was the double layer plaque assay technique described by Varon and Shilo (*15*). The *Bdellovibrio*-prey suspensions were incubated at 30°C for 7 h at 350 rpm. Samples were withdrawn at time 0, 2, 4.5 and 7 h and were plated on nutrient agar (Difco) with a Spiral Plater (Model D, Spiral Systems, Inc., Bethesda, MD). After the plates were incubated for 16 h at 37°C, the colonies formed by the surviving prey bacteria were counted with a Model 500A Bacteria Colony Counter (Spiral Systems, Inc.). Each experiment was replicated at least twice and the average of the data points was plotted. In each experiment, a sample containing only substrate cells in HMB was included as a control. The gram-negative bacteria used as prey were considered to be susceptible to attack by *Bdellovibrio* if the log reduction in the prey population after 7 h was greater than 1.5 log values.

Effects of temperature, pH and predator:prey ratio

To study the effect of temperature on the ability of *Bdellovibrio* to lyse the prey organisms, *E. coli* 2239-69 and *S. enteritidis* Y8P2, the predator:prey suspensions (2:1 ratio, pH 7.4) and the prey controls were incubated at 4, 12, 19, 25, 30 and 37°C at 350 rpm for 24 h. To evaluate the effect of pH on lytic ability, the predator:prey suspensions (2:1 ratio) were incubated at 30°C at 350 rpm for 7 h in HMB adjusted with either O.1 N NaOH or O.1 N HC1 to pH values of 5.6, 6.2, 6.8, 7.4, 8.0 and 8.6. The effect of ratio was studied by incubating the suspensions at ratios of 2:1, 5:1, 10:1, 50:1, 100:1 and 1000:1 of *Bdellovibrio* to prey bacteria in HMB, pH 7.4 at 30°C for 7 h. The level of substrate bacteria was varied while the concentration of *Bdellovibrio* was kept constant at 1 x 10^{10} PFU/ml.

Preparation of bacteria for scanning electron microscopy

Aliquots of *Bdellovibrio*-prey suspensions were withdrawn at times 0, 2 and 4 h and placed on circular glass slides (1 cm in diameter). The bacteria were fixed with 1% glutaraldehyde/0.1 M sodium cacodylate solution, pH 7.3, post-fixed in 2% OsO_4, dehydrated in a graded series of ethanol solutions and critical point dried from liquid CO_2. The samples were coated with a thin layer of gold by DC sputtering and examined by scanning electron microscopy (JOEL, Model No. JSM-840A, Peabody, MA).

RESULTS AND DISCUSSION

The prey organism range of *B. bacteriovorus* 109J is shown in Table 1. The predator was most effective at reducing the levels of both of the *Aeromonas* spp. tested, *E. coli* serotypes O159:H34 and O26:H11, *Pseudomonas* spp., *Salmonella poona, Shigella* spp. and *Yersinia enterocolitica* serotype O:3. The levels of *E. coli* serotype O157:H7 and O157:NM and of *Salmonella* spp. other than *S. poona* were not notably decreased after 7-h incubation with *B. bacteriovorus*.

The present study demonstrates that only certain serogroups of the genera, *Escherichia* and *Salmonella* are susceptible to attack by the predator indicating that bacterial cell wall antigens may be involved in the formation of receptor sites. A specific receptor on the prey cell wall required for attachment has not yet been identified, however, Varon and Shilo (*17*) found that bdellovibrios attached at a much higher rate to rough strain mutants lacking the O-specific side chains but which possessed the complete R-core than to smooth wild-type strains. The absence of certain sugar components of the R-core antigen resulted in decreased attachment indicating that the receptor required for attachment may be located on the R antigen portion of bacterial lipopolysaccharide. Inhibition studies by Houston, Aldridge and Magee et al. (*3*) also suggested that the R antigen is involved in *Bdellovibrio* attachment. Bacteria which possess paracrystalline protein surface layers (S layers) appear to be resistant to attack by *Bdellovibrio* (*5*). A variant of *Caulobacter crescentus*, which lacked an S layer was lysed by

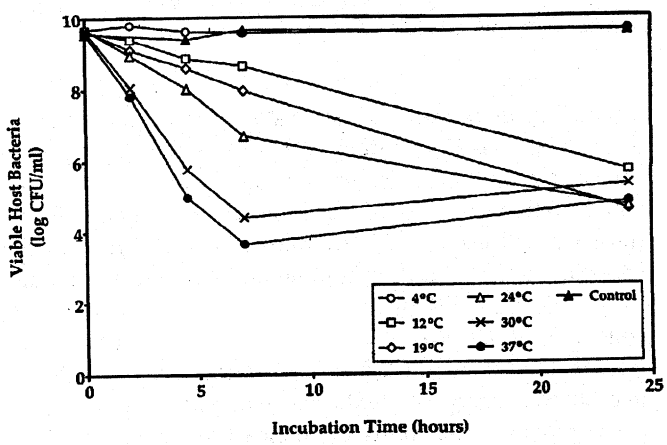

Figure 1. *The effect of incubation temperature on the ability of* B. bacteriovorus *109J to reduce the level of* E. coli *2239-69 at pH 7.4 and using a 2:1 predator to prey ratio.*

B. bacteriovorus 6-5-S and 109J, however the organisms were not able to parasitize the wild-type parental strain.

Bdellovibrio bacteriovorus 109J was most effective in reducing the level of a population of *E. coli* 2239-69 at incubation temperatures of 30 and 37°C (Fig. 1) and least effective at lower temperatures in the range of 4 to 19°C. However, after 24 h incubation of the two-membered culture, there were 3.8 and 5.1 log decreases in the level of prey bacteria at incubation temperatures of 12 and 19°C, respectively. *Salmonella enteritidis* Y8P2 was also incubated with the predator under the diverse temperature and pH conditions to determine if the organism would be susceptible to attack under conditions different than those employed for the prey range studies. There was no notable decline in the level of *S. enteritidis* Y8P2 at any of the incubation temperatures tested even after 24 h of incubation (data not shown). Bdellovibrios are mesophilic organisms with an optimum temperature for activity generally between 25 and 35°C (*15*). Jackson and Whiting (*4*) found an incubation temperature of 30°C to be optimal for activity of *B. bacteriovorus* 109J against *E. coli* K12 as the

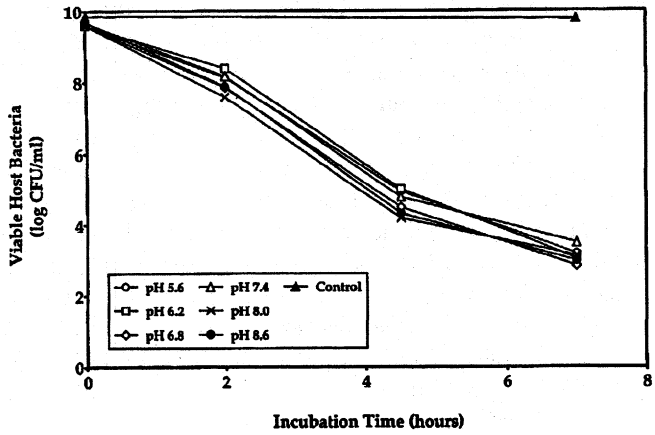

Figure 2. *The effect of pH on the ability of* B. bacteriovorus *109J to reduce the level of* E. coli *2239-69 at 30°C and using a 2:1 predator to prey ratio.*

substrate organism. Whether the predator was active at temperatures above 30°C was not determined. Psychrotrophic bdellovibrios capable of lysing prey bacteria at temperatures of less than 10°C have been isolated, however, the isolates showed increased activity at temperatures above 20°C (*2*).

The activity of *B. bacteriovorus* against *E. coli* 2239-69 was similar in the pH range of 5.6 to 8.6 (Fig. 2). There was no notable reduction in the level of *S. enteritidis* Y8P2 upon incubation with the predator at any of the pH values tested (data not shown). Varon and Shilo (*15*) showed active attachment of *B. bacteriovorus* 109 to *E. coli* B in the pH range of 6 to 9, however, attachment did not occur at pH 5 or below. Jackson and Whiting (*4*) reported that *B. bacteriovorus* 109J was most effective against *E. coli* K12 in the pH range of 6.8 to 7.2. However, although activity was diminished, lysis also occurred at pH 5.2. The effectiveness of the predator at pH values greater than 7.2 was not studied.

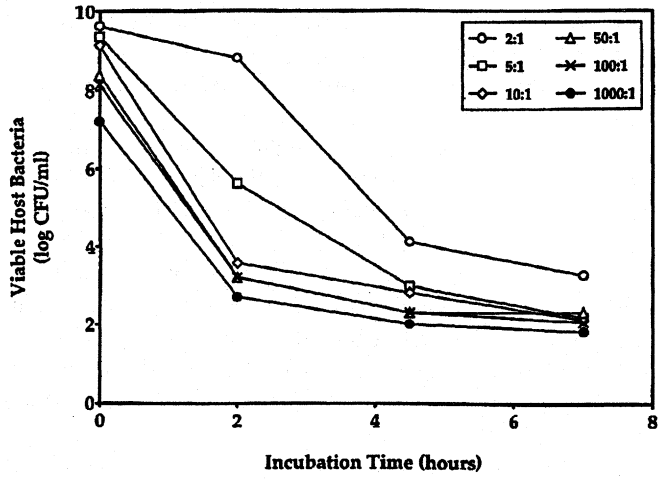

Figure 3. *The effect of varying the predator:prey ratio on the effectiveness of* B. bacteriovorus *109J to reduce the level of* E. coli *2239-69 at 30°C, pH 7.4.*

Increasing the predator:prey ratio resulted in a more rapid decline in the level of *E. coli* 2239-69 (Fig. 3). Using a predator:prey ratio of 2:1, there was a 0.8 log reduction in the prey population after 2 h incubation; however, 10:1 or 50:1 predator to prey ratios resulted in 5.6 and 5.2 log reductions after 2 h, respectively. Under the conditions tested, a ratio of 10:1 appeared to be optimal, resulting in a 7 log decrease in the level of *E. coli* after 7-h incubation. Since *bdellovibrios* encounter susceptible prey bacteria by random collision (*7*), increasing the level of parasite compared to that of prey results in an increased attack rate (*16*). However, Jackson and Whiting (*4*) showed that as the prey density progressively decreases, the probability of the predator infecting the prey organism decreases. *Bdellovibrio bacteriovorus* was most effective in lysing *E. coli* K12 at levels of 5 x 10^9 to 1 x 10^5 *E. coli*/ml. Lysis also occurred at lower concentrations of *E. coli* although not as rapidly (*4*).

Attachment of *Bdellovibrio* to prey bacteria appears to occur very rapidly upon mixing; bdellovibrios were visible by scanning electron microscopy in contact with prey approximately 5 min after mixing (Fig. 4a). *Bdellovibrio* attaches to prey within seconds, although in the initial stages, predator attachment is reversible (*11*). A single sheathed polar flagellum (*14*), which is lost following penetration of the prey bacterium, confers to bdellovibrios the ability to be among the fastest of swimming bacteria. By 2-h incubation, most of the substrate bacteria were preyed upon resulting in the formation of rounded bdelloplasts (Fig. 4b).

Bdellovibrios are widely distributed in nature, therefore, have adapted to survive in a variety of habitats. Consequently, the prey range of different isolates will also vary. Successful use of *Bdellovibrio* to control plant disease has been reported by Scherff (*8*). Application of *B. bacteriovorus* onto soybean inhibited the development of bacterial blight caused by *Pseudomonas glycinea*. However, only 1 of 3 *Bdellovibrio* isolates tested had a significant effect in controlling the development of the disease. The present study demonstrates that *B. bacteriovrorus* 109J is effective in reducing the level of the *Pseudomonas* strains tested. Since *Pseudomonas* is a common spoilage organism, bdellovibrios may potentially be employed on foods to control spoilage.

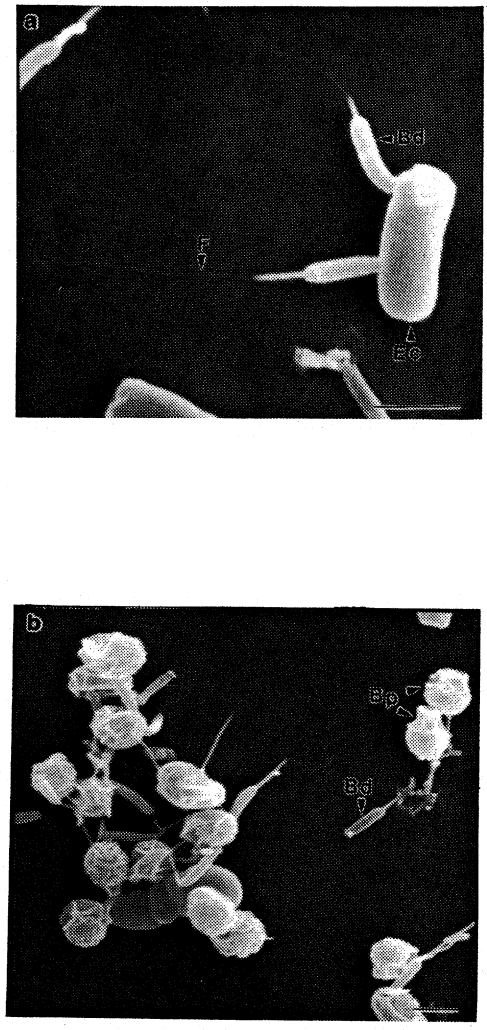

Figure 4. *Scanning electron microscopy view of* B. bacteriovorus *109J mixed with* E. coli *2239-69 (2:1 ratio) and incubated at 30°C, pH 7.4. a) time 0, b) time 2 h. Bd, bdellovibrios; Ec,* E. coli*; Bp, bdelloplast (spheroplasted, infected prey bacterium); F, flagellum. Bars = 1 μm.*

This study demonstrated that bacterial lysis can occur at different predator:prey ratios (Fig. 3). Since the levels of pathogenic and spoilage bacteria in foods will vary, the concentration of bdellovibrios required for effective bacterial lysis will need to be determined for each type of food application. The predator exhibits activity under a moderately wide range of conditions, therefore, it may be used to control bacterial growth in diverse types of foods. Several *Bdellovibrio* isolates may be used in foods in combination in order to encompass a wider range of pathogens and spoilage organisms. The ability of bdellovibrios to attack pathogenic bacteria on meat and other food model systems and on surfaces of equipment used in food processing and preparation is currently under investigation.

ACKNOWLEDGMENTS

The authors thank Ricarda Goins and Benne Marmer for their technical assistance and Peter H. Cooke for processing the bacteria for electron microscopy.

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