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**ISOLATION OF BDELLOVIBRIOS THAT PREY ON  
ESCHERICHIA COLI O157:H7 AND SALMONELLA SPECIES  
AND APPLICATION FOR REMOVAL OF PREY FROM  
STAINLESS STEEL SURFACES<sup>1</sup>**

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Received for Publication November 3, 1995

Accepted for Publication March 26, 1996

**ABSTRACT**

*Predatory bacteria of the genus Bdellovibrio that prey upon Escherichia coli O157:H7 and Salmonella species were isolated from soil and sewage samples. Bdellovibrio isolates 45k (from soil) and 88e (from sewage) attacked and lysed the E. coli and Salmonella strains tested, with log<sub>10</sub> reductions ranging from 2.5 to 7.9 CFU/mL after 7 h incubation, using a two-membered culture system. The predators showed activity against the bacteria within the temperature range of 19 to 37C. Bdellovibrio isolate 45k reduced a population of E. coli dried on stainless steel surfaces by 3.6 log<sub>10</sub> CFU/cm<sup>2</sup> following 24 h contact time and at a 10:1 predator to prey ratio and was also effective in reducing the level of biofilm cells. Bdellovibrios can potentially be utilized for removal of bacteria from surfaces of food processing equipment and possibly for controlling the growth of pathogenic and spoilage bacteria in foods.*

**INTRODUCTION**

*Bdellovibrio* spp., first described by Stolp and Petzold (1962), are predatory microorganisms that attack, penetrate and grow within the periplasm of susceptible gram-negative bacteria. Bdellovibrios are widely distributed in nature and have been isolated from soil, sewage, rivers, man-made water systems (Richardson 1990; Fry and Staples 1976; Klein and Casida 1967; Schoeffield and Williams 1990) and from other aquatic habitats including shellfish (Kelley and Williams 1992; Williams *et al.* 1995b). Williams *et al.* (1995a; 1995b) found bdellovibrios associated with surfaces submerged in natural waters and on which

<sup>1</sup> Mention 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.

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biofilms had formed. The numbers of bdellovibrios firmly attached to surfaces such as oyster shells, glass, steel and titanium increased with time of submersion (1995a). These predators may play a role in reducing the numbers of *Escherichia coli* and other gram-negative bacteria in the environment (Klein and Casida 1967; Mitchell *et al.* 1967). The practice of biological control of plant pathogens using specifically introduced microorganisms has been known for many years (Cook 1993). Scherff (1973) utilized *Bdellovibrio bacteriovorus*, originally isolated from the rhizosphere of soybean roots, to inhibit bacterial blight of soybean caused by *Pseudomonas glycinea*. Biological control techniques are also applicable for control of pathogens in foods. Lactic acid bacteria, commonly used as starter cultures for food fermentations, have also been exploited as “protective cultures” since many metabolic products of these organisms such as organic acids or bacteriocins possess antimicrobial properties (Holzzapfel *et al.* 1995).

Bdellovibrios are capable of infecting and eventually causing lysis of a wide variety of gram-negative bacteria; however, the prey range susceptible to specific *Bdellovibrio* strains varies (Fratamico and Whiting 1995; Schoeffield and Williams 1990; Starr and Stolp 1976; Scherff 1973). Previous studies showed that the prey range of *B. bacteriovorus* 109J did not include *E. coli* O157:H7 and most *Salmonella* species tested in a two-membered system (Fratamico and Whiting 1995). The objectives of the present study were to isolate and test the effectiveness of bdellovibrios with activity against *E. coli* O157:H7 and *Salmonella* in lysing the target organisms and to determine if the predators are capable of reducing the level of the pathogens on stainless steel surfaces.

## MATERIALS AND METHODS

### Bacteria Tested as Prey

The bacterial strains used as prey and their sources are listed in Table 1. The strains were maintained on tryptic soy agar (Difco Laboratories, Detroit, MI) slants stored at 4C.

### Isolation of Bdellovibrios from Soil and Sewage

Bdellovibrios were isolated from soil and sewage samples essentially according to the methods described by Starr and Stolp (1976). Raw sewage was obtained from a local hog slaughter plant and the soil sample was collected from the banks of the Delaware River in Philadelphia. Prey organisms (*E. coli* O157:H7 45753-35 and *E. coli* O157:H7 88.1558) were grown on YGC (1% yeast extract [Difco], 2% glucose, 2% CaCO<sub>3</sub>, 1.5% agar [Difco]) agar plates for 72 h at room temperature. Growth on the plates was removed using a cell scraper (Becton Dickinson and Company, Lincoln Park, NJ) and suspended to a concentration of approximately  $1 \times 10^{10}$  CFU/mL in YP (0.3% yeast extract,

0.06% peptone [Difco] and 0.05M Tris-HCl, pH 7.5) medium diluted 10-fold (YP/10) with sterile H<sub>2</sub>O. Four hundred grams of soil were added to 400 mL of tap water in a 1-L Erlenmeyer flask; the sample was agitated on a gyratory shaker at 200 rpm for 1 h, then centrifuged for 5 min at 2,000  $\times$  g. The supernatant from the soil sample and the sewage sample (100 mL) were filtered sequentially through 0.8 $\mu$ m and 0.45 $\mu$ m filters (cellulose nitrate, Nalge Company, Rochester, NY). Five mL of both the soil and sewage samples were centrifuged at 16,000  $\times$  g for 1 h and 4.5 mL of the supernatant fluid was discarded. The remaining 0.5 mL of samples was vortexed vigorously for 30 s. The double layer plating technique was employed for isolation of *Bdellovibrio* plaques. The bottom and top layers consisted of YP containing 1.2% agar and 0.6% agar, respectively. The soil and sewage samples (0.4 mL) were each mixed with 0.5 mL of prey suspension ( $1 \times 10^{10}$  CFU/mL of YP/10) in 5 mL of top layer agar (kept at 42C) and the mixture was quickly poured on the solidified bottom layer agar. After the top layer was allowed to solidify, the plates were incubated inverted at 30C and the lawns were examined daily for the presence of plaques.

TABLE 1.  
MEASUREMENT OF EFFECTIVENESS OF *BDELLOVIBRIO* ISOLATES 45K AND 88E AT REDUCING THE LEVEL OF *ESCHERICHIA COLI* AND *SALMONELLA* SPECIES

Substrate Bacteria	Source <sup>a</sup>	Log <sub>10</sub> Reduction in Prey Population After 7h <sup>b,c</sup>	
		Soil Isolate (45k)	Sewage Isolate (88e)
<i>S. enteritidis</i> 5-1952	UPVS	3.12	3.24
<i>S. seftenberg</i> Pro 16855	UPVS	2.50	5.17
<i>S. poona</i>	AMS	4.30	5.11
<i>S. typhimurium</i> 14028	ATCC	4.82	3.95
<i>S. dublin</i> 15480	ATCC	4.39	5.00
<i>E. coli</i> 45753-35 (O157:H7)	FSIS	5.67	6.39
<i>E. coli</i> 88.1558 (O157:H7)	ECRC	6.02	2.56
<i>E. coli</i> C984 (O157:H7)	CDC	3.83	6.19
<i>E. coli</i> A9218-C1 (O157:H7)	CDC	4.33	4.91
<i>E. coli</i> (O157:NM)	ECRC	5.18	6.19
<i>E. coli</i> 2239-69 (O26:H11)	CDC	6.23	7.92

<sup>a</sup> UPVS, University of Pennsylvania Veterinary School; AMS, Agricultural Marketing Service, Gastonia, NC; ATCC, American Type Culture Collection; FSIS, Food Safety and Inspection Service; ECRC, *E. coli* Reference Center, University Park, PA; CDC, Centers for Disease Control.

<sup>b</sup> Log<sub>10</sub> value of number of prey bacteria at time 0 h minus log<sub>10</sub> value of number of prey bacteria at time 7 h. Control samples (no *Bdellovibrio* added) showed no notable change in CFU/mL after 7 h.

<sup>c</sup> The difference in the log<sub>10</sub> values of the number of surviving prey bacteria of the replicate experiments was within 0.5 log<sub>10</sub> units.

### **Purification and Maintenance of Bdellovibrios**

Material from individual plaques was obtained using a sterile pipet tip and suspended in 1 mL of YP/10. In order to obtain a purified *Bdellovibrio* isolate, plaque assays were repeated several times, using the same prey bacteria as were used for the initial plating. After several transfers, material from an isolated plaque was added to prey bacteria ( $1 \times 10^{10}$  CFU/mL) in 5 mL of HEPES metal buffer (HMB), pH 7.4 (Fratamico and Whiting 1995) in a 50 mL Erlenmeyer flask and was incubated with aeration at 250 rpm for 32 h at 30C. The isolates were propagated weekly by adding 2 mL of the lysate to 8 mL of prey bacteria in dilute nutrient broth (Fratamico and Whiting 1995) and incubating at 30C for 16 h at 250 rpm. The *Bdellovibrio* isolates were maintained in liquid culture at 4C as lysates (progeny *Bdellovibrio* and lysed prey bacteria).

### **Determination of Prey Range**

The ability of the *Bdellovibrio* isolates to attack various bacteria used as prey was tested as described previously (Fratamico and Whiting 1995). Briefly, following propagation of the *Bdellovibrio* isolates in 200 mL of prey suspension (Fratamico and Whiting 1995) (isolates 88e and 45k using *E. coli* O157:H7 88.1558 and 45753-35, respectively), the bdellovibrios and prey were mixed in 10 mL of HMB at a ratio of 2:1 ( $1 \times 10^{10}$  plaque forming units (PFU)/mL *Bdellovibrio*: $5 \times 10^9$  CFU/mL prey). The two-membered cultures were incubated at 30C for 7 h at 350 rpm, and samples were withdrawn at time 0, 2, 4.5 and 7 h. Appropriate dilutions were made in 0.1% peptone and the samples were plated on nutrient agar with a Spiral Plater (Model D, Spiral Systems, Inc., Bethesda, MD). After incubation for 16 h at 37C, colonies formed by surviving prey bacteria were counted with a Model 500A Bacteria Colony Counter (Spiral Systems, Inc.). In each experiment, a control consisting of only prey bacteria in HMB was included.

### **Determination of Optimum Temperature for Activity of Bdellovibrios**

To study the effect of temperature on the ability of the *Bdellovibrio* isolates, 88e and 45k, to lyse the prey bacteria, the two-membered cultures (2:1 ratio) were incubated at 4, 12, 19, 25, 30 and 37C at 350 rpm. Samples for plating were withdrawn at 0, 2, 4.5, 7 and 24 h.

### **Activity of Bdellovibrios on *E. coli* on Stainless Steel Surfaces**

The effectiveness of *Bdellovibrio* isolate 45k in reducing the level of bacteria dried on stainless steel surfaces was tested. Overnight cultures of *E. coli* O157:H7 strain 45753-35 and serotype O26:H11 strain 2239-69 were suspended in HMB at a concentration of  $1 \times 10^{10}$  CFU/mL and 100  $\mu$ L was spread evenly

over most of the surface of 1 in. square stainless steel sections. The bacteria were allowed to dry on the surfaces and 100  $\mu\text{L}$  of HMB containing *Bdellovibrio* 45k at concentrations of either  $5 \times 10^{10}$  PFU/mL (resulting in 1:5 *E. coli* to *Bdellovibrio* ratio) or  $1 \times 10^{11}$  PFU/mL (1:10 *E. coli* to *Bdellovibrio* ratio) was added. At time 0 h the bacteria were removed from the stainless steel by wiping sequentially with 3 calcium alginate swabs (Type 2 Calgiswab, Spectrum Laboratories, Inc., Houston, TX), placing the swabs in sterile 0.1% peptone, vortexing to dislodge the bacteria and plating dilutions onto nutrient agar. The other inoculated stainless steel sections were incubated at 30C for 5 h then processed as described above. Incubation for the remaining stainless steel sections was continued at 19C for 19 additional hours before testing (24 h samples). As controls, stainless steel sections containing only *E. coli* and HMB were tested at time 0, 5 and 24 h.

The ability of isolate 45k to lyse biofilm bacteria was also examined. *E. coli* 45753-35 was grown overnight at 37C in nutrient broth. Five hundred microliters of the culture was added to a sterile petri dish containing 25 mL of nutrient broth and the stainless steel chips. The dishes were left at room temperature for 48 h to allow the formation of a biofilm on the stainless steel. The chips were then lifted carefully with sterile tweezers and excess liquid was removed by pressing one of the edges against several paper towels. They were then placed in separate sterile petri dishes, and either 500  $\mu\text{L}$  of HMB (controls) or 500  $\mu\text{L}$  of a suspension of *Bdellovibrio* isolate 45k in HMB ( $1 \times 10^9$  PFU/mL) was placed on the stainless steel chips so as to cover the entire top surface. The samples were incubated in a humid chamber at 30C for 24 h. One set of samples (both control and test samples) was processed at time 0 by removing the liquid on the stainless steel chips with a pipet, then drying the surface with calcium alginate swabs in order to recover most of the bacteria. The samples were diluted in 0.1% peptone and plated onto nutrient agar. The second set of samples was processed in the same manner after 24 h incubation.

### Scanning Electron Microscopy (SEM)

Following 24 h of incubation, the stainless steel chips (control and test samples) were lifted with sterile tweezers and gently immersed in 5 mL of 1% glutaraldehyde-0.1M imidazole chloride solution. They were left overnight at room temperature, then washed in 0.1M imidazole buffer and dehydrated in 50% ethanol and absolute ethanol. They were critical point dried, mounted on large stubs with colloidal silver adhesive and sputter coated with gold for secondary electron imaging SEM.

## RESULTS AND DISCUSSION

### Isolation of Bdellovibrios

Several *Bdellovibrio*-like plaques were visible on lawns of *E. coli* approximately 7 days after preparing the plates with the sewage and soil samples and the presence of bdellovibrios was confirmed by phase contrast and scanning electron microscopy. In the initial isolation, many contaminating bacteria and phage plaques as well as plaques formed by bdellovibrios were present on the plates. Phage plaques usually appear within 12 to 24 h and do not increase notably in size. Individual large plaques appearing after several days' incubation of the plates were selected, examined microscopically for the presence of bdellovibrios and the material was diluted and used for a second plaque assay. The process was repeated several times until contaminating bacteria were no longer visible. *Bdellovibrio* isolates 45k and 88e were isolated from soil and sewage samples, respectively.

Differences within the genus *Bdellovibrio* have been assessed through phenotypic examination, phage-susceptibility studies and 16S ribosomal RNA, genetic and serological analyses (Burnham and Conti 1984; Donze *et al.* 1991; Kramer and Westergaard 1977; Seidler *et al.* 1972; Althausen *et al.* 1972). Members of the genus, consisting of *B. bacteriovorus*, *B. stolpii*, *B. starrii* and marine *Bdellovibrio* species, possess the ability to attack and lyse susceptible prey bacteria. Prey-independent mutants, capable of growth in the absence of prey (axenic growth) and facultative mutants which are able to grow in the presence or absence of prey bacteria have been isolated (Burnham and Conti 1984; Shilo and Bruff 1965). Cotter and Thomashaw (1992) identified a genetic locus, *hit*, apparently associated with the host-independent phenotype.

### Prey and Temperature Range for the *Bdellovibrio* Isolates.

*Bdellovibrio* strains show prey specificity among various genera and species of gram-negative bacteria. Although, enteric bacteria are suitable target organisms, many bdellovibrios may not efficiently attack and lyse different bacterial species and serotypes (Fratamico and Whiting 1995; Schoeffield and Williams 1990; Starr and Stolp 1976). *Bdellovibrio* strains isolated from marine habitats require sodium chloride or sea water for growth. It has been found that, generally, bacteria found in marine environments are more suitable prey for marine bdellovibrios than are bacteria of terrestrial origin (Taylor *et al.* 1974). In the present study, both *Bdellovibrio* isolates (45k and 88e) attacked and lysed the *Salmonella* and *E. coli* strains tested (Table 1). However, there were differences in the effectiveness of the two isolates in lysing the various strains. For example, isolate 45k reduced a population of *S. seftenberg* Pro 16855 by  $\log_{10}$  2.5, whereas, the bacterial population decreased by  $\log_{10}$  5.17 after 7 h using

isolate 88e. Both isolates were notably effective against *E. coli* 2239-69. The temperature range for activity of the *Bdellovibrio* isolates was between 19 and 37C with optimal activity at temperatures of 25-37C (data not shown). Continued incubation of the cocultures for 24 h at the various temperatures did not result in an increase in reduction of log<sub>10</sub> CFU compared to 7 h incubation.

#### Use of *Bdellovibrio* to Reduce the Level of *E. coli* O157:H7 on Stainless Steel

Stainless steel is a material widely used in the food processing industry. Since bacteria adhering to processing equipment are potential sources of contamination for foods that come in contact with the surfaces, effective sanitation procedures are required. Food material containing pathogenic bacteria may dry on surfaces of utensils and equipment used in food processing. Bacteria on stainless steel or

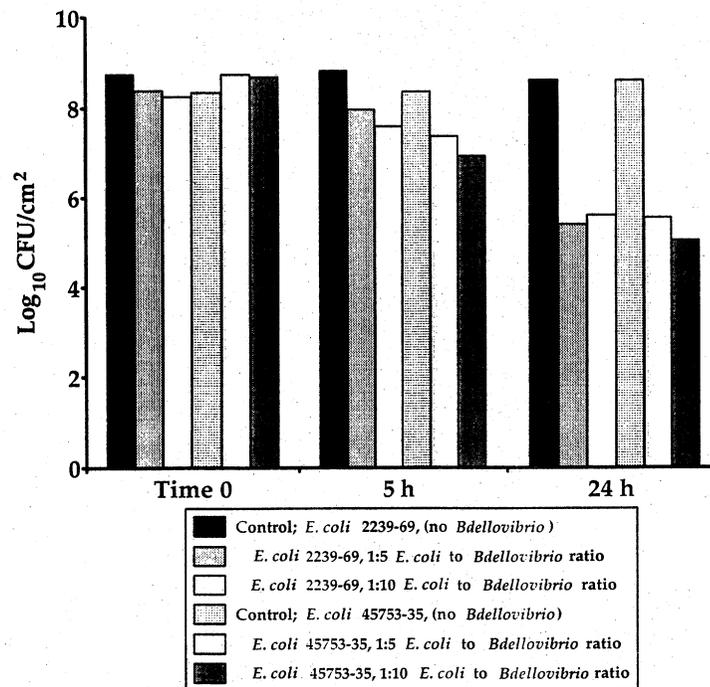


FIG. 1A. USE OF *BDELLOVIBRIO* ISOLATE 45K FOR REDUCTION IN THE LEVEL OF *E. COLI* DRIED ON STAINLESS STEEL

The samples, treated and not treated with *Bdellovibrio*, were incubated at 30C for 5 h and at 19C for an additional 19 h. Bacteria were removed from the stainless steel surfaces at time 0, 5 and 24 h and dilutions were plated onto nutrient agar.

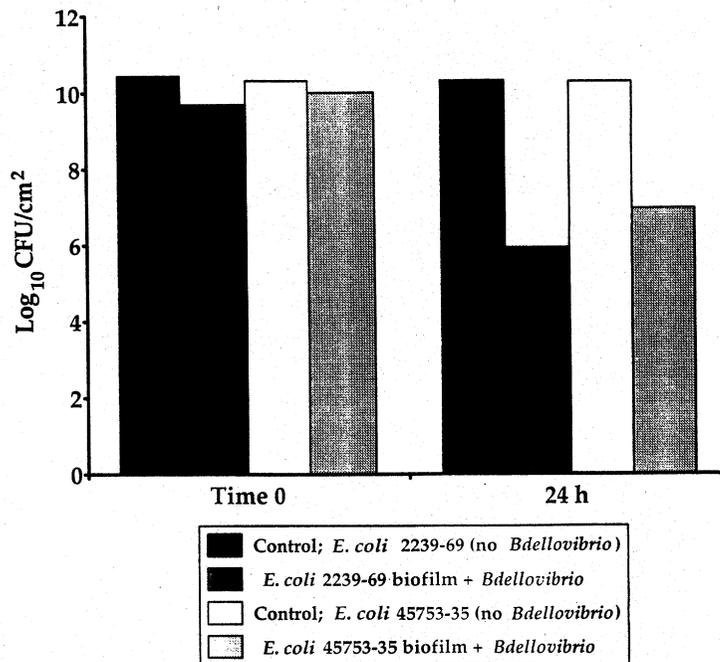


FIG. 1B. USE OF *BDELLOVIBRIO* ISOLATE 45K FOR REDUCTION IN THE LEVEL OF *E. COLI* BIOFILMS ON STAINLESS STEEL

The samples, treated and not treated with *Bdellovibrio*, were incubated at 30C for 24 h. Bacteria were removed from the stainless steel surfaces at time 0 and 24 h and dilutions were plated onto nutrient agar.

other surfaces such as rubber or Teflon®, if left undisturbed, may also form biofilms, thus rendering them more resistant to sanitizing agents (Mosteller and Bishop 1993). However, surfaces in contact with foods such as meat are usually used, washed and sanitized regularly, therefore, rendering it difficult for biofilms to form. Biofilms will form on surfaces in contact with liquids such as dairy equipment or floor drains in processing plants (Zottola and Sasahara 1994). Generally, sanitizers employed in food plant operations include chlorine-, iodine-based or quarternary ammonium compounds or combinations of detergents and sanitizers (Troller 1983). In recent years, however, environmental concerns over the use of chemicals as sanitizers have been raised. A novel approach is the use of predatory bacteria which are harmless to humans as an alternative to chemical sanitizers for removal of bacteria on food processing equipment. In the present study, the ability of *Bdellovibrio* 45k to lyse *E. coli* dried onto stainless steel surfaces or after formation of biofilms was studied.

The levels of *E. coli* strains 2239-69 and 45753-35 dried on stainless steel were reduced by 3.00 and 3.19  $\log_{10}$  CFU/cm<sup>2</sup>, respectively, following 24 h contact with bdellovibrios and at a 1:5 prey:predator ratio (Fig. 1A). *E. coli* 45753-35 was reduced by 3.64  $\log_{10}$  CFU/cm<sup>2</sup> by 24 h at a 1:10 prey:predator ratio. SEM micrographs also showed a decrease in the number of *E. coli* on the stainless steel (Fig. 2). There was no notable change in the number of culturable

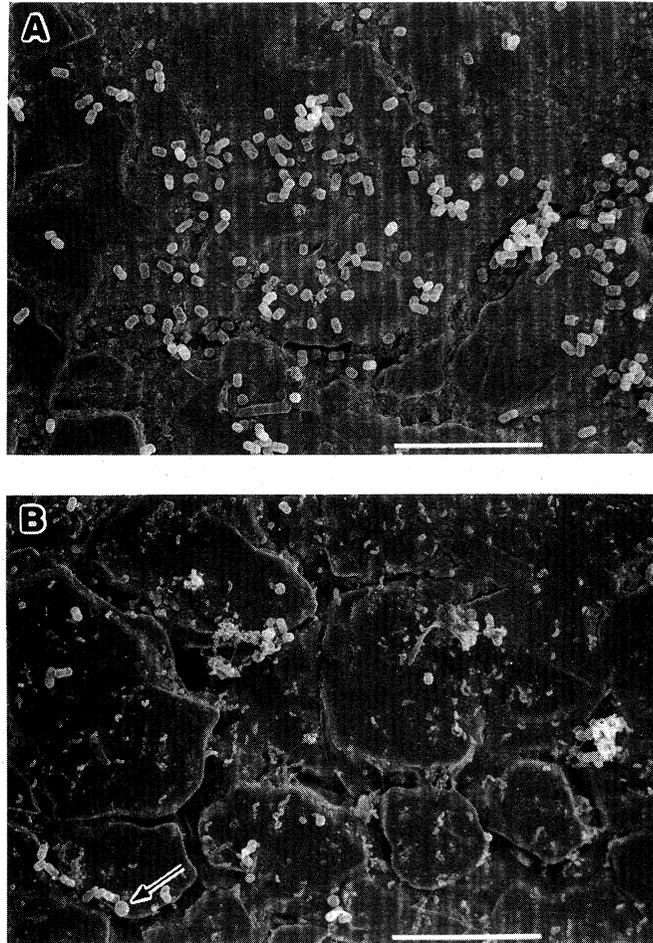
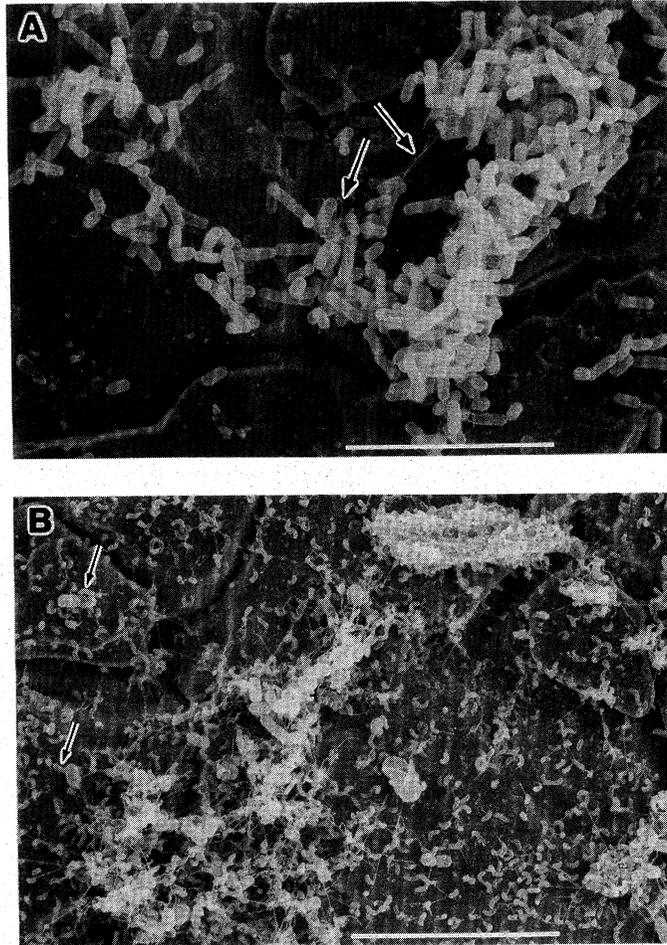


FIG. 2. SCANNING ELECTRON MICROGRAPHS SHOWING *E. COLI* DRIED ON STAINLESS STEEL SURFACES UNTREATED (A) AND TREATED (B) WITH *BDELLOVIBRIO* ISOLATE 45K

The samples were incubated at 30C for 24 h before processing for SEM. Arrow points to a bdelloplast (spheroplasted, infected prey bacterium). Bar = 10 $\mu$ m.

*E. coli* on stainless steel sections in which the application of bdellovibrios was omitted (Controls, Fig. 1A and 1B), indicating that the bacteria remained viable for 24 h in HMB. *Bdellovibrio* 45k was effective in reducing the level of *E. coli* biofilm cells. By 24 h, *E. coli* 2239-69 was reduced by 3.76 log<sub>10</sub> CFU/cm<sup>2</sup> and



**FIG. 3. SCANNING ELECTRON MICROGRAPHS SHOWING *E. COLI* BIOFILMS ON STAINLESS STEEL SURFACES UNTREATED (A) AND TREATED (B) WITH *BDELLOVIBRIO* ISOLATE 45K**

The samples were incubated at 30C for 24 h before processing for SEM. Large arrows point to exopolysaccharide matrix and small arrows point to bdellovibrios attacking *E. coli*. Bar = 10µm.

*E. coli* 45753-35 was reduced by 3.05 log<sub>10</sub> CFU/cm<sup>2</sup> (Fig. 1B). Extracellular matrix and microcolonies were visible by SEM in *E. coli* biofilms formed on stainless steel (Fig. 3). Bdellovibrios attacking *E. coli* cells were observed and the number of *E. coli* appeared to be reduced compared to control biofilms which did not receive bdellovibrios (Fig. 3).

Isolation of prey-independent mutants (Shilo and Bruff 1965), facultative (Diedrich *et al.* 1970) and halophilic strains (Taylor *et al.* 1974; Sánchez Amat and Torrella 1989) and of *Bdellovibrio* capable of forming stable bdellocysts with increased resistance to temperature, desiccation and disruption (Tudor and Conti 1977) has been reported. This study demonstrates that isolation of prey-dependent bdellovibrios from different environmental sources which have activity against the desired target organisms can be achieved. Furthermore, bdellovibrios can potentially be used to control the growth of pathogens on food contact surfaces such as stainless steel.

#### ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. John Tudor and Dr. Richard Whiting for helpful discussions and thank Dr. Daniel Solaiman and Dr. Eugene Weinberg for critical review of the manuscript.

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