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Formation of Stable Bdelloplasts as a Starvation-Survival Strategy of Marine Bdellovibrios

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Several wild-type isolates of marine bdellovibrios formed stable bdelloplasts when they infected gram-negative bacterial prey under certain culture conditions. Synchronous predator-prey cultures and low nutrient concentrations increased the yield of stable bdelloplasts. The bdellovibrio cells retained in the stable bdelloplasts showed a high survival capacity in nutrient-depleted saline solution (10% viable *Bdellovibrio* cells after 3 months at 25°C), whereas *Bdellovibrio* attack-phase cells kept under the same starvation conditions lost viability more quickly (1% viable cells after 48 h). The addition of yeast extract to a stable bdelloplast suspension induced lysis of the bdelloplasts and release of motile infecting attack-phase *Bdellovibrio* cells. Other substances, such as free amino acids, protein hydrolysates, NH_4^+, carbohydrates, and organic amines, did not induce such a release. Stable bdelloplasts were highly hydrophobic and had a lower endogenous respiration rate than attack-phase cells. In general, stable bdelloplasts were almost as sensitive to temperature changes, desiccation, sonication, tannic acid, and Triton X-100 treatment as attack-phase cells. Electron microscopy of stable bdelloplasts did not reveal any extra cell wall layer, either in the bdelloplast envelope or in the retained *Bdellovibrio* cells, unlike the bdellocysts of the soil bacterium *Bdellovibrio* sp. strain W. We propose that formation of stable bdelloplasts is a survival strategy of marine bdellovibrios which occurs in response to nutrient- and prey-poor seawater habitats.

Bacteria belonging to the genus *Bdellovibrio* have been isolated from a variety of habitats, including soil (15), rivers (1), estuarine waters (21), seawater, and solar salt concentration ponds (13). The presently known *Bdellovibrio* life cycle consists of the following two phases: a motile, vibroid, extracellular attack phase, during which the predators look for prey and do not divide; and a spiral growth phase in the periplasmic space of gram-negative bacteria (10). Many aspects of the ecology of the bdellovibrios remain to be elucidated, including the survival strategy of aquatic forms.

Previous starvation-survival studies on *Bdellovibrio* species have been performed by using washed suspensions of attack-phase cells in nutrient-depleted media. Studies of freshwater and marine strains have shown a very high mortality rate for this *Bdellovibrio* extracellular form (3, 6).

Bdellovibrio sp. strain W, a soil isolate, forms resting cells in the periplasmic space of prey cells. These bdellocysts are more resistant than attack-phase cells to changes in various environmental factors, particularly desiccation (16). In part, the resistance of the bdellocysts is probably enhanced by the extra layer of amorphous material that surrounds the resting cells (17). This survival strategy is not surprising for a soil *Bdellovibrio* strain that is subjected to desiccation periodically. Recent studies have shown that viable bdellovibrios can be recovered from 2-year-old air-dried stored soil, suggesting that there is some type of resting stage in other soil bdellovibrios (2). However, for aquatic forms, particularly marine forms, no comparable resting stages have been described previously.

Aquatic environments in general and marine environments in particular have discontinuities (patchiness) in their concentrations of nutrients. Usually, free waters have a very low concentration of organic matter and hence a very low concentration of actively growing heterotrophic bacteria. The majority of these microorganisms exist under various degrees of starvation (5, 7). In such environments *Bdellovibrio* cells have fewer opportunities for finding prey. In addition, the interruption of *Bdellovibrio* intraperiplasmic growth has been reported when mixed predator-prey cell cultures are diluted (19). On the other hand, there are microenvironments where the concentrations of nutrients are higher and sustain active growth of heterotrophic bacteria. In this case, a higher concentration of gram-negative prey cells might support an active population of bdellovibrios (20).

In our search for the starvation-survival strategy of marine bdellovibrios, we examined life cycle stages other than the attack phase. The role of the formation of stable bdelloplasts as a survival mechanism in the normal life cycle of marine bdellovibrios is described in this paper.

MATERIALS AND METHODS

***Bdellovibrio* and host strains.** In this study we used five marine *Bdellovibrio* isolates (strains CP1, CP2, CP3, CP4 and BS12) and four gram-negative prey strains (*Pseudomonas* sp. strain UM1, *Vibrio parahaemolyticus* UM1, *Vibrio alginolyticus* UM2, and *Vibrio splendidus* UM1). *Bdellovibrio* sp. strain BS12 was isolated from a solar salt pond; all of the other *Bdellovibrio* and prey strains used in this work were isolated from Mediterranean Sea water and have been described elsewhere (13).

A spontaneously derived streptomycin-resistant mutant of *Bdellovibrio* sp. strain CP1 was obtained by using the method of Seidler and Starr (14); this method was adapted to marine bacteria by using *V. parahaemolyticus* UM1 as prey and NBS/10 medium (13).

Culture conditions. All of the media contained a basal saline solution, which contained (per liter) 24 g of NaCl, 7 g

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of $MgSO_4 \cdot 7H_2O$, 5.3 g of $MgCl_2 \cdot 6H_2O$, 0.7 g of KCl, and 1.25 g of $CaCl_2$.

Pseudomonas sp. strain UM1 was routinely cultured in TRIPLE (tryptone-yeast extract) medium, which contained 2.3 g of tryptone and 1.2 g of yeast extract in 1 liter of basal saline solution; the pH was adjusted to 7.4 with NaOH. The vibrios were cultured either in tryptone-soy broth (TSB), which contained (per liter) 15 g of tryptone, 5 g of soytone, and 30 g of NaCl (pH 7.5), or in NBS/10 medium. All of the culture media were purchased from Oxoid Ltd. Chemicals were obtained from either E. Merck AG or Panreac and were analytical grade.

Actively motile *Bdellovibrio* suspensions were routinely obtained in SST (basal saline solution containing 1.57 g of Tris hydrochloride per liter; pH 7.4). Washed suspensions of *V. parahaemolyticus* UM1 that were grown previously in tryptone-soy broth were inoculated with a predator strain taken from a previous lysate from the same prey. Active cultures of *Bdellovibrio* sp. strain BS12 were obtained in NBS/10 medium by using *V. alginolyticus* UM2 as prey because this predator strain developed faster under the conditions which we used. Excess prey cells and bdelloplasts were removed from the culture lysates by low-speed centrifugation (1,500 × *g*, 4 min), and the supernatants, which contained released bdellovibrios, were further cleaned by filtration through a membrane (pore size, 1.2 μm; Millipore Corp.).

Synchronous single-cycle cultures were prepared by mixing suspensions of SST-washed, freshly released bdellovibrios cells (centrifuged twice at 15,000 × *g* for 15 min) with previously washed prey substrate cells. A multiplicity of infection (MOI) of 1.5 to 2 predator cells per substrate cell was used. Bacterial concentrations were estimated turbidimetrically by using calibrated curves. Generally, cultures containing 0.8×10^9 to 1×10^9 prey cells per ml and 1.5×10^9 to 2×10^9 predator cells per ml were established. In order to remove unattached bdellovibrio cells, each mixed culture was centrifuged after 1 to 1.5 h (3,000 × *g*, 10 min), the supernatant was discarded, and the sediment was suspended in the same volume of SST. By that time, the culture had all of the cells that could be observed with microscopy in the bdelloplast stage.

Starvation protocol. Either bdelloplasts or attack-phase *Bdellovibrio* cells were washed twice in SST and then suspended in the same solution to a final concentration of 10^7 PFU/ml; 50-ml portions of the suspensions were maintained in 100-ml flasks at 25°C in a rotatory shaker at 100 rpm. Viability was estimated at different times by using the double-layer agar technique, in which *V. parahaemolyticus* UM1 was the prey, NBS/10 medium prepared with 0.8% Oxoid agar no. 3 was the bottom layer, and 0.6% agar was the top layer.

Resistance to environmental factors. For studies of resistance of *Bdellovibrio* attack-phase cell suspensions and stable bdelloplasts to different temperatures, desiccation, and sonication we used the methods of Tudor and Conti (16), with the modifications described below. To determine heat resistance, 0.5-ml samples containing 10^7 bdellovibrio cells or stable bdelloplasts per ml were placed in tubes with 4.5 ml of SST at different temperatures. The suspensions were maintained at constant temperatures for 15 min and then cooled and plated to test for viability. To test desiccation resistance, 0.1 ml of a 10^9-PFU/ml cell or bdelloplast suspension in SST was placed in a drying chamber with silica gel, and a slight vacuum was applied for 24 h. Then 2 ml of SST was added, the culture was shaken vigorously, and the suspension was plated to test for predator viability. Resistance to sonication was determined by subjecting 5-ml suspensions containing 2×10^8 to 3×10^8 bdellovibrio cells or bdelloplasts per ml to succesive cycles of sonication (30-s bursts, 15-s cooling cycles) in a Sonic Dismembrator (Dynatech Corp.) at a relative output energy of 0.4. During sonication the samples were kept at a constant temperature in a ice bath. Sensitivity to surfactants was studied by adding Triton X-100 (final concentration, 0.2%) to a suspension containing 3×10^8 to 5×10^8 PFU of *Bdellovibrio* cells or bdelloplasts per ml. Samples were removed at various times and plated to determine viability. Resistance to tannic acid was determined by diluting a *Bdellovibrio* suspension to a concentration of 3×10^7 PFU/ml in 0.1% tannic acid in basal saline solution, either at pH 3 or at pH 6.3 (a higher pH induced precipitation of the tannic acid solution). Samples were removed at different times and plated to determine viability.

Alkaline phosphatase activity. Alkaline phosphatase activity was determined by mixing 0.3 ml of 0.1 M Tris hydrochloride solution (pH 8.9) containing 5 mM $MgCl_2$ and 10 mM 4-nitrophenylphosphate (Boehringer Mannheim Biochemicals) with 0.2 ml of a sample. The mixture was incubated at 30°C for 30 min, and the reaction was stopped by adding 0.2 M Na_2CO_3. Optical density was read at 410 nm to measure enzyme activity both in the supernatant and in the cell extracts of *Bdellovibrio* cells or bdelloplasts. A cell extract was obtained after 0.5 ml of a culture was diluted with 5 ml of SST and the resulting preparation was centrifuged at 15,000 × *g* for 15 min. The supernatant was kept for further analysis, and the pellet was suspended in 5 ml of SST and subjected to sonic disruption (Dynatech Sonic Dismembrator; relative output power, 0.5; 12 30-s cycles with 15-s cooling intervals). The sample tube was maintained in an ice bath and centrifuged at 15,000 × *g* for 15 min before the supernatant (extract) was assayed for enzyme activity.

Oxygen uptake. Oxygen consumption by either actively motile *Bdellovibrio* attack-phase cells or 1-week-old stable bdelloplasts was determined with an oxygen monitor (Yellow Springs Instrument Co.) by using 3-ml samples of preparations containing 2×10^9 to 3×10^9 PFU/ml. Cell suspensions were washed twice in SST.

Electron microscopy. *Bdellovibrio* cells were fixed for negative staining with neutralized formaldehyde (final concentration, 4%) for 24 to 48 h at 4°C. Fixed suspensions were washed twice with distilled water and stained with 0.5% uranyl acetate for 1 min on Formvar-coated grids.

Ultrathin sections were obtained from cell concentrates that were fixed and stained by using a modification of the method of Kellenberger et al. (4). Samples (10 ml) were prefixed with 1 ml of 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2). After centrifugation at 15,000 × *g* for 15 min, the resulting pellet was suspended in 1 ml of OsO_4 fixative containing 1% tryptone. The sample was incubated overnight at room temperature, diluted with cacodylate buffer, and centrifuged. The resulting pellet was suspended in 2% agar in cacodylate buffer, and this preparation was cut into small blocks. These blocks were dehydrated with a graded series of ethanol, embedded in Epon and included in gelatin capsules. Thin sections were obtained by using a Ultracut-Reichter-Yung microtome and were mounted on copper grids. The resulting sections were stained with uranyl acetate (5%, 5 min) and lead citrate (9).

TABLE 1. Percentage of *Pseudomonas* sp. strain UM1 cells that remain as stable bdelloplasts after 5 days of incubation of a mixed culture containing these prey cells and *Bdellovibrio* sp. CP1 in SST at 25°C

Conditions for pregrowth of the prey[a]		% of stable bdelloplasts compared with originally infected cells	
Temp (°C)	Growth phase	Undiluted culture	Diluted culture[b]
25	Exponential	2	33
25	Initial stationary	41	ND[c]
15	Exponential	0.6	28
15	Initial stationary	19	33

[a] *Pseudomonas* cells were precultured in TRIPLE medium at either 15 or 25°C and harvested at different phases of growth.
[b] A bdelloplast suspension that was 1 h old was diluted 1/15 in SST.
[c] ND, Not determined.

RESULTS

Formation of stable bdelloplasts. The first observations of the formation of prey cell bdelloplasts that did not lyse after the usual 3- to 4-h period following their formation (stable bdelloplasts) were made during experiments in which washed cells of *Pseudomonas* sp. strain UM1 taken from the stationary growth phase were used as substrates for *Bdellovibrio* cells. In these first experiments, the proportion of cells that were transformed into stable bdelloplasts and the stability of the bdelloplasts were both highly erratic. A careful search for the factors that promoted the formation of stable bdelloplasts and promoted stability was undertaken.

A basic requirement for obtaining a good yield of stable bdelloplasts was to wash the prey cells free of the previous growth medium before the two-member culture was established. For example, stable bdelloplasts did not form in a mixed predator-prey culture in NBS/10 medium. An MOI of one to two bdellovibrio cells per prey cell and good synchronization of the infection process promoted the formation of a high proportion of stable bdelloplasts in mixed cultures. The use of a low MOI (e.g., 1 to 10 bdellovibrio cells per 100 prey cells) produced successive lytic cycles in mixed cultures. The accumulation of nutrients and cell debris from the first cycles appeared to affect the prey cells so that the phenomenon of bdelloplast stabilization was not observed.

Table 1 shows the percentages of stable bdelloplasts obtained by using prey cells cultured under different conditions. The size of the prey cells seemed to be a possible factor in determining the efficiency of formation of stable bdelloplasts because higher yields of stable bdelloplasts were obtained with smaller cells. For organisms in the same phase of growth, cells grown at 15°C were bigger than cells grown at 25°C. Thus, when cells grown at 15°C were used, a lower percentage of stable bdelloplasts was formed than when cells grown at 25°C were used (Table 1). Independent of prey cell size, when a suspension of 1-h-old stable bdelloplasts (1 h after the attack of prey cells by *Bdellovibrio* cells) was diluted 1/15 in SST, the proportion of stable bdelloplasts increased. Thus, cell density in a mixed culture also had an effect on the yield of stable bdelloplasts, suggesting that one or more substances released into the menstruum may play a key role in the process of bdelloplast rupture.

When *Pseudomonas* sp. strain UM1 cells from the exponential phase of a culture grown in TRIPLE medium at 25°C (these cells were bigger than early- and late-stationary-phase cells) were washed and used as prey, good synchronicity of infection was obtained (after 15 min all prey cells had been transformed into bdelloplasts). As judged by microscopic counts, 98% of these bdelloplasts lysed after about 3 to 3.5 h, giving rise to very active *Bdellovibrio* progeny cells. On the other hand, if *Pseudomonas* cells were harvested from the stationary phase, bad synchronicity was observed (bdellovibrios did not attach to many of the prey cells), but 15% of the bdelloplasts formed did not lyse and remained stable. Late-exponential- to early-stationary-phase cells were still suitable for good synchronicity, and mixed cultures showed a substantial (up to 40%) proportion of stable bdelloplasts that did not lyse after 3.5 h. The stable bdelloplasts formed under these conditions were used in the majority of our experiments.

In order to determine the permeability of prey cells during attack by *Bdellovibrio* cells, alkaline phosphatase activity in synchronized cultures was measured in the supernatants and in the whole-cell fractions. We found that *Bdellovibrio* sp. strain CP1 lacked this enzyme, just as other bdellovibrios do (8). *Pseudomonas* sp. strain UM1 is alkaline phosphatase positive, and probably maintains the enzyme in the periplasm. When prey cells used in a mixed culture were collected in the exponential phase of growth, the enzyme quickly started to disappear from the bdelloplasts and to be detected in the supernatant (Fig. 1A). A high proportion of the initial enzyme activity was released before the disruption of the bdelloplasts (3 to 3.5 h after attack). On the other hand, when stationary-phase prey cells were attacked by *Bdellovibrio* cells, very little of the initial enzymatic activity appeared in the medium during the process of bdelloplast formation. Because a small fraction of bdelloplasts lysed under these conditions, after 3.5 h a low level of enzyme activity was detected in the supernatant (Fig. 1B). The enzyme that remained in the stable bdelloplasts was probably degraded, leading to a loss of activity in the cell fraction during the process of bdelloplast stabilization.

***Bdellovibrio* survival capacity in nutrient-depleted media: attack-phase cells and bdelloplasts.** The survival capacity of bdellovibrio cells in bdelloplasts was clearly much higher than the survival capacity of free cells (Fig. 2). The addition of a complex nutrient mixture to a bdelloplast suspension (4 ml of TRIPLE medium to 10 ml of suspension) induced the lysis of stable bdelloplasts and the release of an average of two actively motile predator cells per bdelloplast. The newly released bdellovibrio cells lost viability at the typical high rate for the attack-phase cells, whether they were maintained in the activating medium or they were washed and suspended in SST (Fig. 2). The decrease in survival showed a lag when nutrients were added, probably because the viability of the bdellovibrio cells was temporarily maintained by the added nutrients. *Bdellovibrio* sp. strain CP1 is a prey-dependent strain that cannot divide in TRIPLE medium. A streptomycin-resistant derivative of *Bdellovibrio* sp. strain CP1 had to be used in these experiments in order to prevent the growth of the remaining unlysed *Pseudomonas* sp. strain UM1 prey cells when activating nutrients were added. When samples were plated, the streptomycin was diluted enough to prevent activity against prey strain *V. parahaemolyticus* UM1, which is a streptomycin-sensitive strain.

Induction of the release of bdellovibrio cells from stable bdelloplasts. The effect of the addition of different substrates on the induction of bdelloplast lysis was determined by microscopic observation and by *Bdellovibrio* plate counting before and after induction of stable bdelloplast suspensions with these substrates. *Bdellovibrio* sp. strain CP1 (Sm^r) was

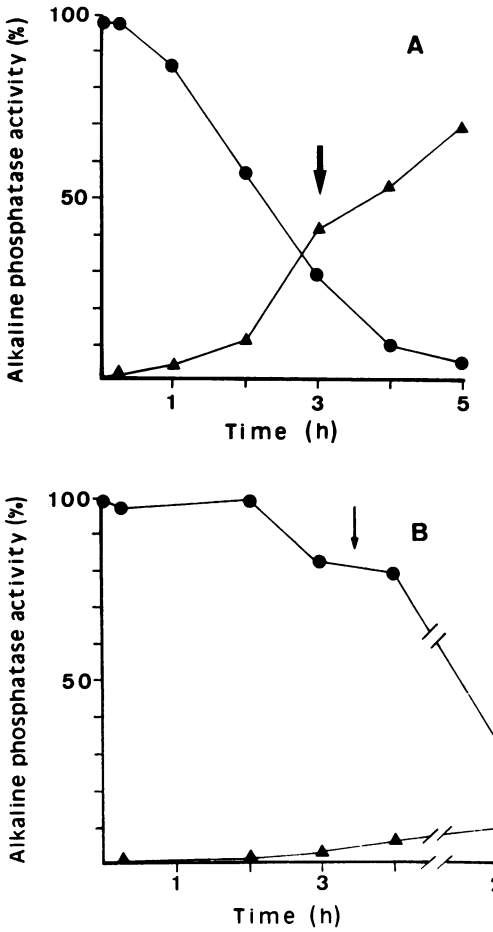

FIG. 1. Alkaline phosphatase activities in supernatants (▲) and cells (●) of a synchronous culture containing *Bdellovibrio* sp. strain CP1 and *Pseudomonas* sp. strain UM1. *Pseudomonas* cells were pregrown in TRIPLE medium at 25°C until the exponential phase (A) or the stationary phase (B) before a mixed culture in SST was established. (A) The arrow indicates the time when the majority of the bdelloplasts were ruptured. (B) The arrow indicates the time when only a small fraction of the bdelloplasts were ruptured.

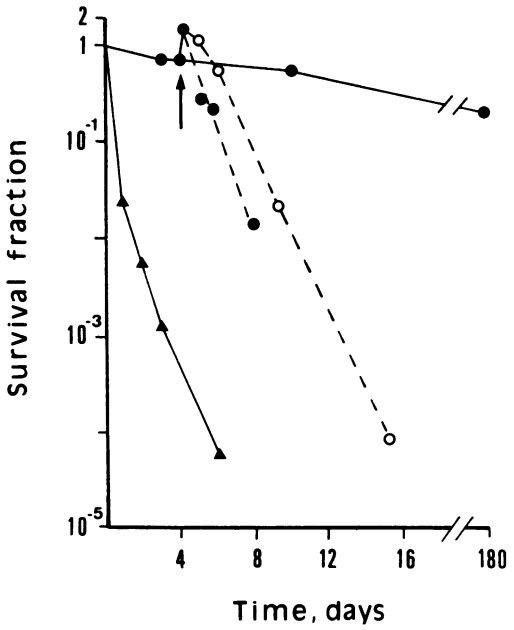

FIG. 2. Survival of *Bdellovibrio* sp. strain CP1 attack-phase cells (▲) and *Bdellovibrio* sp. strain CP1 Sm^r cells within *Pseudomonas* sp. strain UM1 stable bdelloplasts (●). To induce the release of attack-phase bdellovibrio cells from the bdelloplasts, 4 ml of TRIPLE medium was added to 10 ml of a 5-day-old bdelloplast suspension (arrow). The activating medium contained 500 μg of streptomycin sulfate per ml to prevent proliferation of nonlysed surviving *Pseudomonas* sp. strain UM1 cells. The newly released bdellovibrio cells were maintained in the activating medium (●) or were washed and suspended in SST (○).

used in all of these experiments so that streptomycin could prevent proliferation of Sm^s unlysed *Pseudomonas* cells after addition of the different substrates. Yeast extract was the only addition which activated the bdelloplasts and induced release of *Bdellovibrio* attack-phase cells. Casamino Acids (0.5%), glutamine (0.1 M), glutamic acid (0.1 M), NH_4Cl (0.05 M), ATP (90 μM), glucose (1%), mannose (1%), putrescine (1 μM), spermine (1 μM), a mixture of the four nitrogenous bases present in DNA (each at a concentration of 63 μM), and a combination of ATP, nitrogenous bases, and amino acids failed to induce release of attack-phase cells.

When either streptomycin or 5 mM KCN was added to a stable bdelloplast suspension of *Pseudomonas* sp. strain UM1 infected by wild-type *Bdellovibrio* sp. strain CP1 before yeast extract was added, activation of stable bdelloplasts was inhibited. These data suggest that protein synthesis and respiratory-dependent energy synthesis are both necessary for the activation process.

Resistance of stable bdelloplasts to environmental factors. Stable bdelloplasts were slightly more resistant than *Bdellovibrio* attack-phase cells to elevated temperature treatments (Fig. 3). A 15-min exposure to 37°C reduced the level of viable free *Bdellovibrio* cells to 49%, whereas the viability of a stable bdelloplast suspension was almost unaffected (92%

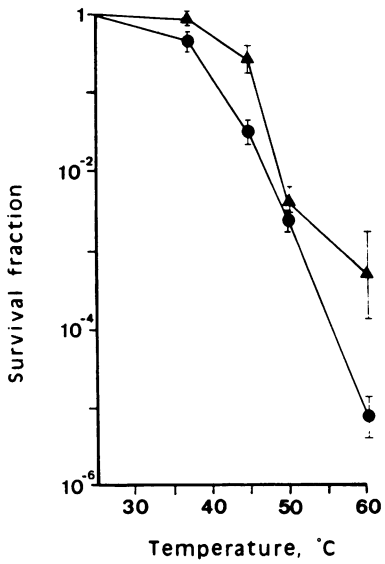

FIG. 3. Survival capacities of attack-phase *Bdellovibrio* sp. strain CP1 cells (●) and stable bdelloplasts of *Pseudomonas* sp. strain UM1 (▲) after different temperature treatments.

TABLE 2. Effect of sonication on suspensions of *Bdellovibrio* sp. strain CP1 attack-phase cells and stable bdelloplasts of infected *Pseudomonas* sp. strain UM1 cells[a]

No. of sonication cycles	Relative % of surviving bdellovibrio cells	
	Attack-phase cells	Stable bdelloplasts
0	(100)	(100)
1	127 ± 2	163 ± 40
3	72 ± 8	163 ± 42
5	73 ± 1	215 ± 68
7	83 ± 5	125 ± 30

[a] Samples were sonicated as described in the text and were plated to determine viability. The results are the means of three independent experiments.

viable cells). A 15-min exposure to 45°C killed 96.5% of attack-phase cells and only 87.5% of stable bdelloplasts.

Free bdellovibrio cells and stable bdelloplasts were both very sensitive to desiccation. The fractions of surviving cells after the desiccation protocol were 8.14×10^{-7} and 6.9×10^{-7} for attack-phase cells and stable bdelloplasts, respectively.

The increase in viable cell concentration in a free cell suspension after a single sonication cycle (Table 2) may have been due to mechanical dispersion of adherent viable cells. The following six sonication cycles decreased the level of viable cells to 59% compared with the first cycle. In the case of bdelloplast suspensions, microscopy after the first rounds of sonication showed that the effect of sonic treatment was mainly to disperse the aggregates of the highly hydrophobic stable bdelloplasts up to fifth cycle, when there was an increase in the level of viable cells because of the rupture of the bdelloplasts. The decrease in viability after the sixth cycle (58% compared with the previous cycle) indicated that the *Bdellovibrio* cells released from bdelloplasts were as sensitive as attack-phase cells to sonication.

Bdellovibrio cells prematurely released by sonication were not motile. Subsequently motility of the bdellovibrios appeared after 3 to 3.5 h of incubation in media containing yeast extract. The bdellovibrios artifically liberated from bdelloplasts could also synthesize flagella and complete their life cycle when they were inoculated into the top layer of a double-layer agar plate used to count viable bdellovibrio cells.

Bdellovibrio cells in stable bdelloplasts were more sensitive than attack-phase cells to the nonionic detergent Triton X-100 (Table 3). This detergent dispersed the aggregates of bdelloplasts and solubilized their envelopes, releasing the detergent-sensitive bdellovibrio cells. Electron microscopy

FIG. 4. Electron micrograph of a negatively stained *Bdellovibrio* sp. strain CP1 cell released from a stable bdelloplast with Triton X-100 (0.2%, 1 min). Bar = 0.3 μm.

revealed that these cells did not have flagella and were the same size as attack-phase cells (Fig. 4). The tendency to form aggregates and accumulate at the surface of the medium also suggests that the surfaces of stable bdelloplasts are hydrophobic. Tannic acid was very active in killing both attack-phase cells and bdellovibrio cells in bdelloplasts (Table 4). If the tannic acid solution was not neutralized, its low pH was an additional detrimental factor.

Oxygen uptake. A comparison of the respiration rates of a 7-day-old stable bdelloplast suspension and a suspension of *Bdellovibrio* attack-phase cells showed that 10^9 attack-phase cells consumed 0.85 nmol of O_2 per min and that an equally concentrated stable bdelloplast suspension consumed 0.53 nmol of O_2 per min. Considering that an average stable bdelloplast of *Pseudomonas* sp. strain UM1 formed with stationary-phase cells contained two bdellovibrio cells, the endogenous respiration rate of the bdellovibrio cells in bdelloplasts was roughly 30% that of the same number of attack-phase bdellovibrio cells. An additional indication of the endogenous respiration capacities of stable bdelloplasts

TABLE 3. Resistance to Triton X-100 of *Bdellovibrio* sp. strain CP1 attack-phase cells and stable bdelloplasts of infected *Pseudomonas* sp. strain UM1 cells[a]

Time of treatment (min)	Relative % of surviving bdellovibrio cells	
	Attack-phase cells	Stable bdelloplasts
0	(100)	(100)
0.5	117 ± 8	126 ± 3
30	58 ± 5	22 ± 10
60	60 ± 8	17 ± 8
90	52 ± 8	2.7 ± 3

[a] Cell suspensions in SST supplemented with 0.2% Triton X-100 were incubated at 25°C for various times and subsequently were plated to determine viability. The results are the means of three independent experiments.

TABLE 4. Resistance of *Bdellovibrio* sp. strain CP1 attack-phase cells and stable bdelloplasts of infected *Pseudomonas* sp. strain UM1 to tannic acid[a]

Concn of tannic acid in SST (%)	pH	Time (min)	Relative % of surviving bdellovibrio cells	
			Attack-phase cells	Stable bdelloplasts
0	7.4	30	100	100
0.1	3	10	$<10^{-4}$	$<10^{-4}$
0.1	6.3	10	4.3×10^{-2}	1.6
0.1	6.3	30	4.6×10^{-2}	1.9×10^{-2}

[a] Samples were incubated under the conditions indicated and were plated to determine viability.

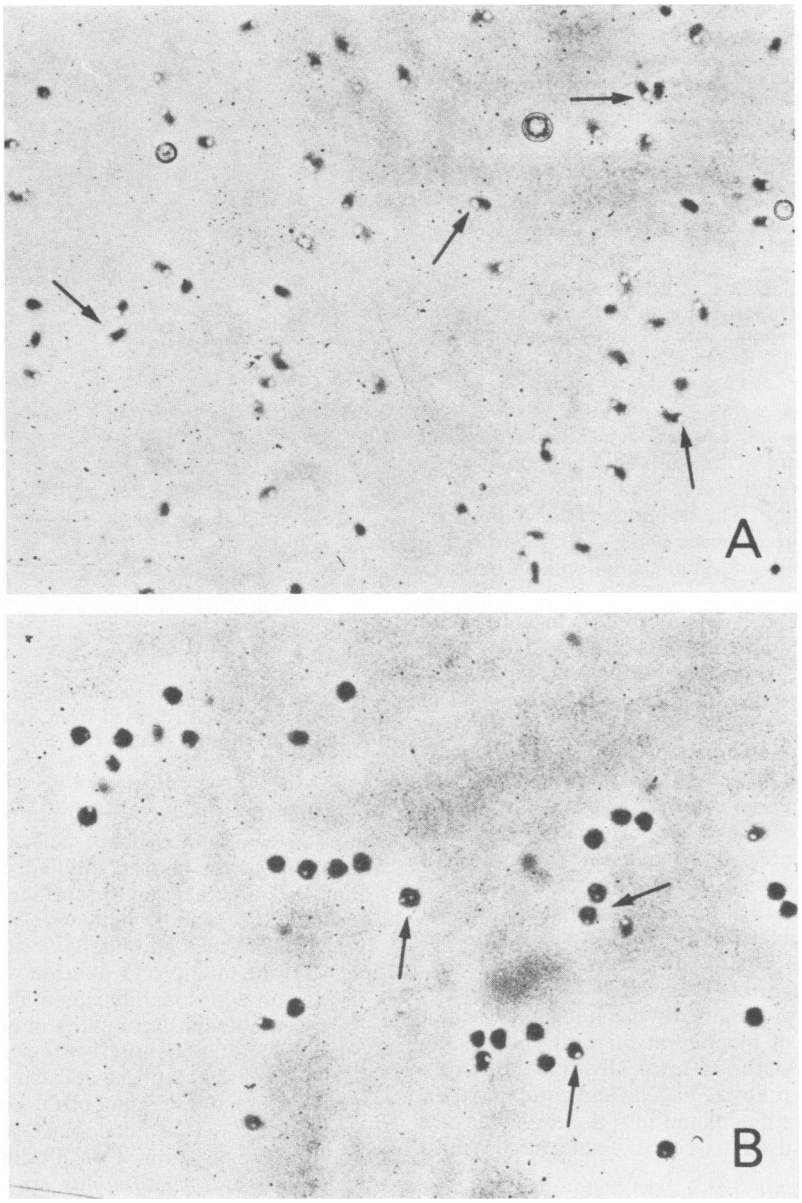

FIG. 5. Phase-contrast micrograph of *Bdellovibrio* sp. strain CP1 attack-phase cells (A) or a 3-week-old *Pseudomonas* sp. strain UM1-*Bdellovibrio* sp. strain CP1 stable bdelloplast suspension (B) incubated with 0.02% INT for 30 min. The arrows indicate intracellular spots of formazan.

and attack-phase cells was obtained by incubating suspensions of both types in the presence of 0.02% *p*-iodonitrotetrazolium violet (INT) (Sigma). After 30 min of incubation, granules of formazan were observed associated with the cells of an actively motile suspension of recently released attack-phase bdellovibrio cells (Fig. 5A). Even after 3 weeks we observed two granules of formazan per stable bdelloplast when INT was added (Fig. 5B).

Ultrastructure of stable bdelloplasts. Thin sections of 1-week-old stable bdelloplasts containing one or two *Bdellovibrio* cells revealed that the outer membrane of the prey cells became the stable bdelloplast wall (Fig. 6A and B). The cytoplasmic membrane and outer membrane of the bdellovibrio cells contained in the bdelloplasts were clearly evident, and no additional cell layers were visible. The cytoplasm of the prey cells appeared to be almost completely degraded. The nucleoids of the predator cells filled much of the cytoplasm of the prey cells, and some inclusion bodies were present in the nucleoid area. This kind of inclusion bodies was seldom observed in attack-phase bdellovibrio cells (data not shown) but was more frequent and larger in bdellovibrio cells contained in bdelloplasts.

Formation of stable bdelloplasts in other *Bdellovibrio*-prey systems. Table 5 shows a number of *Bdellovibrio*-prey systems in which the formation of stable bdelloplasts has been observed by using the experimental protocol described above for *Bdellovibrio* sp. strain CP1 and *Pseudomonas* sp. strain UM1. Mixed cultures were established in SST with a MOI of two predator cells per prey cell, and both predator and prey cells were washed free of any remaining nutrients

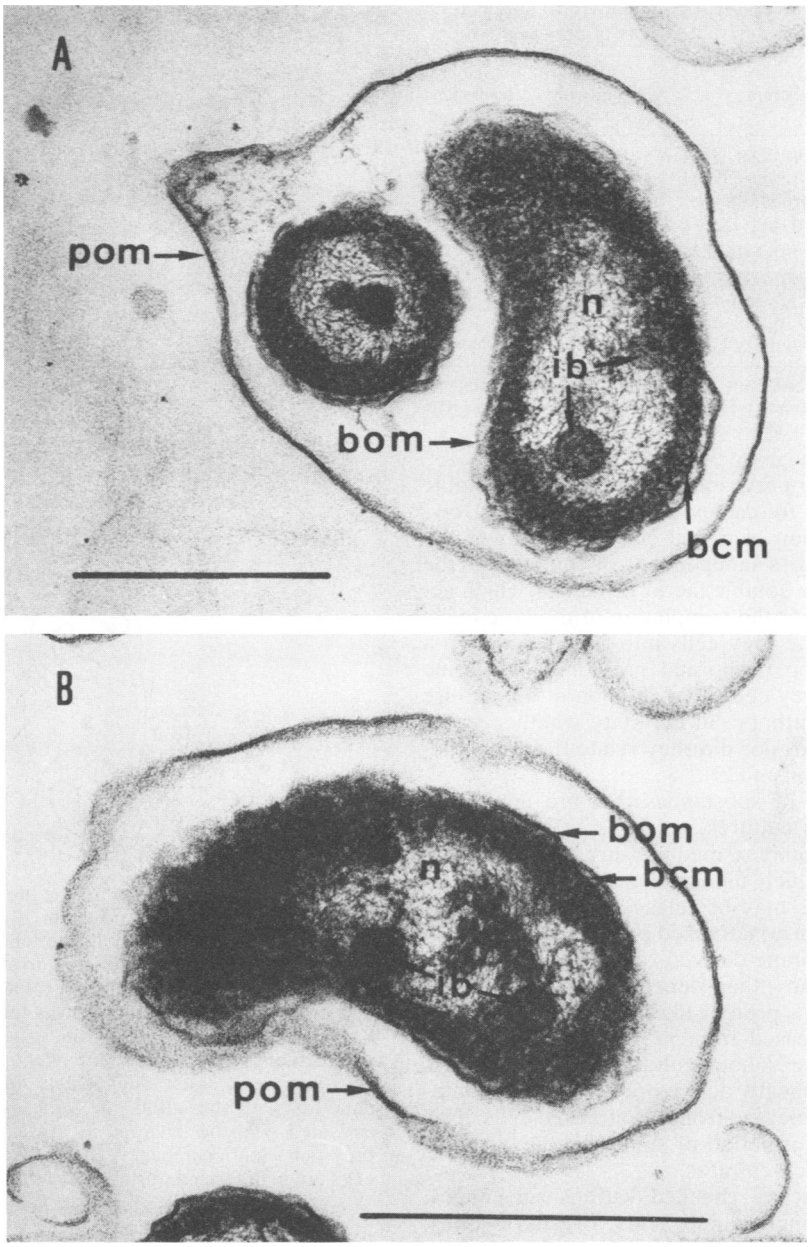

FIG. 6. Electron micrographs of thin sections of 1-week-old stable bdelloplasts of *Pseudomonas* sp. strain UM1 infected with *Bdellovibrio* sp. strain CP1. pom, Prey outer membrane; bom, *Bdellovibrio* outer membrane; bcm, *Bdellovibrio* cytoplasmic membrane; ib, inclusion body; n, nucleoid area. Bars = 0.5 μm.

from previous cultures. The prey cells used in the mixed cultures were harvested from the beginning of the stationary phases of previous cultures. All of the *Bdellovibrio* strains tested formed stable bdelloplasts with at least one prey strain.

DISCUSSION

We describe a survival mechanism of marine bdellovibrios that includes the formation of stable bdelloplasts that retain viable *Bdellovibrio* progeny in a state of relatively reduced metabolic activity for long periods of time. The onset of this mechanism is affected by the physiological environment of the *Bdellovibrio* cells. In our work we tried to simulate as closely as possible the situation that marine bdellovibrios may face in nature, especially concerning low nutrient concentrations and the influence that this may have on prey cells. Maintaining a low nutrient concentration in mixed *Bdellovibrio*-prey batch cultures is not an easy task because lysis of the prey cells enriches the medium with soluble nutrients, membranes, and walls fragments. In order to avoid the influence of such compounds on *Bdellovibrio* intraperiplasmic growth, we prepared synchronous single-cycle cultures with high MOIs.

The formation of stable bdelloplasts depends on prey cell size, cell concentration, and the type of nutrients present in or released into the medium during the mixed culture stage.

TABLE 5. Capacity of several marine bdellovibrio strains to form stable bdelloplasts on different strains of prey bacteria[a]

Bdellovibrio strain	Prey strain	Pre-growth medium	Stable bdelloplast formation
CP1	*V. parahaemolyticus* UM1	NBS/10	+
CP1	*V. parahaemolyticus* UM1	TSB	−
CP1	*V. alginolyticus* UM2	TSB	+
CP1	*V. splendidus* UM1	NBS/10	+
CP4	*Pseudomonas* sp. strain UM1	TRIPLE	+
CP2	*Pseudomonas* sp. strain UM1	TRIPLE	+
BS12	*Pseudomonas* sp. strain UM1	TRIPLE	+
CP3	*Pseudomonas* sp. strain UM1	TRIPLE	−
CP3	*V. parahaemolyticus* UM1	TSB	+

[a] In all experiments the prey cells were grown at 25°C until the beginning of the stationary phase. Mixed cultures were always established in SST with an MOI of 2 predator cells per prey cell.

As a general rule, smaller prey cells are converted into stable bdelloplasts at a higher frequency than larger cells. However, the fact that dilution of mixed cultures improves the yield of stable bdelloplasts independent of the size of the prey cells suggests that a soluble factor or factors which act as the signal necessary for the completion of the lytic cycle may be released from the prey cells into the medium. In a previous paper cessation of normal growth of a marine *Bdellovibrio* strain on prey cells after dilution of the culture was reported, but the authors did not state whether stable bdelloplasts were formed, nor did they relate the phenomenon to a survival mechanism (19).

Many different kinds of substances that are present in mixed *Bdellovibrio*-prey cultures, particularly if they have been established in a complex medium, are candidates for the unknown factors which induce the rupture of bdelloplasts. These substances may be released from the bdelloplasts themselves or from nonattacked prey cells and include such materials as membrane-derived oligosaccharides (12). Moreover, our results show that, depending on the phase of prey cell growth, even a protein like the enzyme alkaline phosphatase can be released from prey cells after *Bdellovibrio* attack. In nonsynchronous cultures there are always nonattacked and metabolically active prey cells that eventually use the substances released from the attacked cells. This could explain why the formation of stable bdelloplasts has not been observed in such cultures.

Although in our work we checked various substances, including sugars, ammonia, amino acids, organic amines, and others, the nature of the factor which hampers the stabilization of bdelloplasts remains unknown. Because yeast extract induces the activation of the bdellovibrio cells contained in stable bdelloplasts, it seems that this compound either contains or facilitates the activity of the lytic factor. *Bdellovibrio* sp. strain W bdellocysts can be induced to germinate by glutamine and other amino acids and by NH_4^+ ions (18). Nevertheless, these compounds and many others do not induce activation of stable bdelloplasts from marine bdellovibrios. After the addition of yeast extract, 2.5 to 3 h is necessary for the disruption of stable bdelloplasts at 25°C. This time period seems to be necessary for the synthesis of the flagella and probably other factors involved in the release of active bdellovibrio cells from bdelloplasts.

The survival capacity of *Bdellovibrio* sp. strain CP1 attack-phase cells in saline solution is very low and similar to the survival capacity described previously for other *Bdellovibrio* strains (3, 6). The bdellovibrio cells retained inside stable bdelloplasts survive for substantially longer periods of time, and after 3 months a survival level of 10% can be still detected. This survival mechanism has not been described previously, and there are important differences between this survival mechanism and the survival mechanism of bdellocysts of *Bdellovibrio* sp. strain W. For example, after activation, each stable bdelloplast releases more than one progeny cell, depending on the initial size of the prey cells, whereas a germinating bdellocyst gives rise to a single attack-phase cell (18). Another difference is that stable bdelloplasts are not notably resistant to environmental factors, such as desiccation. It seems logical that a soil *Bdellovibrio* strain would require a resting stage that is resistant to desiccation, an environmental stress which is common in soils but which may not be as critical to marine bacteria.

The low resistance of stable bdelloplasts to desiccation, as well as heat, may result from the lack of specific differentiation in the cell wall layers of the bdelloplasts compared with bdellocysts (17). It is common that starvation survival of

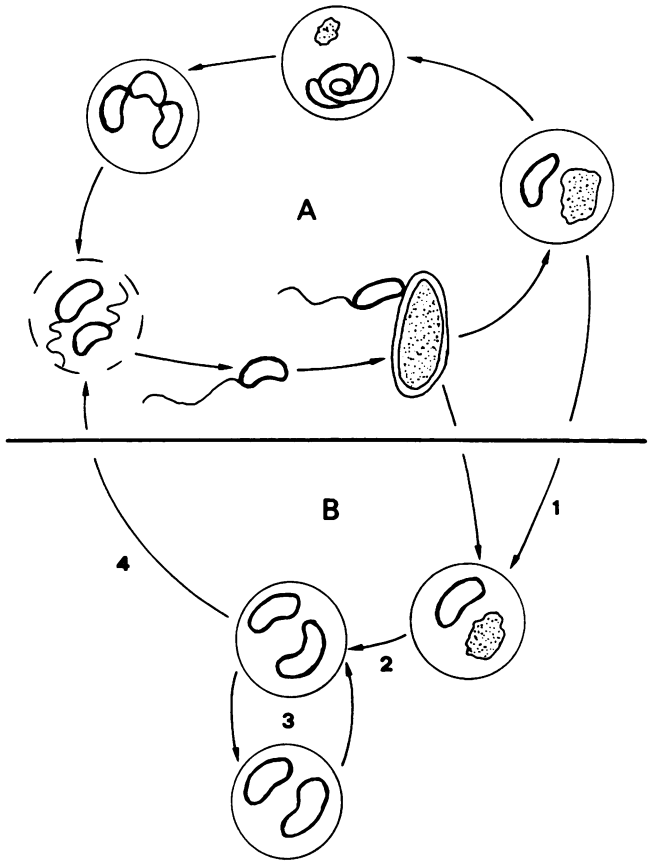

FIG. 7. Proposed model for the life cycle of marine *Bdellovibrio* species suggested by the results of this work. (A) Growth of *Bdellovibrio* cells on a prey in nutrient- and prey-rich medium. (B) Adaptation of a *Bdellovibrio* strain to starvation conditions. In stage 1, a bdelloplast is transported to a nutrient-poor area, or the nutrients where the bdelloplast was formed are exhausted. In stage 2, the *Bdellovibrio* cell(s) degrades the cytoplasmic contents of the prey cell but remains inside the bdelloplast. In stage 3, *Bdellovibrio* cells survive in stable bdelloplasts for long periods of time under conditions of starvation. In stage 4, the bdellovibrio cells are activated, and the bdelloplasts are ruptured when a nutrient- and prey-rich situation appears. The flagella of the activated bdellovibrio cells are synthesized during this period.

marine bacteria takes place in a cell stage that is structurally similar to growing cells (11).

The inclusion bodies that are frequently found in contact with the nuclear material of bdellovibrio cells retained in stable bdelloplasts have been observed previously in bdellocysts and some attack-phase cells of other *Bdellovibrio* strains (17). It has been suggested that these inclusion bodies are reserve materials, but electron microscope images of these bodies are not typical of any of the known reserve materials found in procaryotes (e.g., poly-β-hydroxybutyrate, polyphosphates, glycogen). The chemical nature of the inclusion bodies remains unknown, but because they are not as easily observed in attack-phase cells as in resting cells, they may function in the survival and activation of bdellovibrio cells retained in bdellocysts. The same function has been proposed for the inclusion bodies of *Bdellovibrio* sp. strain W bdellocysts (17). This material may sustain the endogenous respiration observed in stable bdelloplasts.

The survival mechanism described in this paper seems to be general among marine bdellovibrios because stable bdelloplasts have been found in all of the strains that have been checked, although in some strains the stable bdelloplasts are formed only with certain prey strains. Many marine bacteria are adapted to intermittent growth dynamics, growing actively in nutrient-rich microenvironments and developing starvation-survival strategies in nutrient-poor situations (5). Our results indicate that there is a similar intermittent mode of growth of bdellovibrios in marine waters (Fig. 7). *Bdellovibrio* cells are able to multiply in areas where nutrients, and consequently gram-negative bacteria, are abundant. However, if infected prey cells are transported to a nutrient-poor area, the lytic cycle of the *Bdellovibrio* cells cannot be completed, and stable bdelloplasts are then formed. The activation of stable bdelloplasts depends on a signal of unknown nature; however, this signal is probably present when environmental conditions allow the maintenance and growth of prey bacteria. Stable bdelloplasts are highly hydrophobic, and this could also be a characteristic that facilitates the adsorption of bdelloplasts onto surfaces where the growth of prey cells may be enhanced.

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