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Differentiation After Premature Release of Intraperiplasmically Growing *Bdellovibrio bacteriovorous*

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Bdellovibrio bacteriovorous attacks and penetrates other gram-negative bacteria, creating a growth chamber termed a bdelloplast. We have found that exposing the bdelloplasts to EDTA, followed by treatment with a lytic enzyme concentrate derived from bdellovibrio cultures, prematurely released the intraperiplasmically growing bdellovibrios at any time during their growth cycle. Upon release, the growth-form bdellovibrios terminated any initiated rounds of DNA synthesis and differentiated into motile attack-form cells. The ability of growth-form cells to synthesize DNA appears to depend upon an initiation signal that is not received until about 60 min after attack. Each subsequent round of DNA synthesis by the growing bdellovibrio filaments seems to require an additional initiation signal that is provided by their intraperiplasmic environment. Differentiation included fragmentation into multiple progeny cells to a degree proportional to the extent of intraperiplasmic growth. This differentiation could be performed totally at the expense of cellular reserves. The significance of these data to an understanding of the regulation of differentiation in bdellovibrios is discussed.

The life cycle of the predatory bacterium *Bdellovibrio bacteriovorous* consists of an obligate alternation between two distinct cellular types, an attack-phase cell and a growth-phase cell. Predation is initiated by a small, flagellated, somewhat curved, rod-shaped cell incapable of independent proliferation, which attacks, kills, and enters into the periplasmic space of a gram-negative prey bacterium. After penetration, the intraperiplasmic bdellovibrio undergoes a remarkable physiological and morphological differentiation into a nonflagellated cell that elongates as a coiled nonseptate filament, growing at the expense of prey cell material. It is only during this growth period that DNA replication can occur in wild-type *B. bacteriovorus*. When intraperiplasmic growth ceases, the filament undergoes a second differentiation, forming multiple septa and flagella and fragmenting into motile attack-phase cells that exit the remains of the spent prey bacterium. Each of these cells then begins the search for prey to initiate a new round of the cycle (for recent reviews, see references 12 and 14).

This dimorphic, obligate, sequential transformation between an extracellular, motile attack phase and an intraperiplasmic, nonmotile, proliferating growth phase makes bdellovibrios an attractive model for examining the control of procaryotic differentiation. However, the conditions or effectors responsible for growth initiation or cell division are largely uninvestigated. This is primarily because both of these events occur after bdellovibrio penetration of the prey cell, which complicates the experimental manipulation of their growth environment.

The isolation and characterization of the lytic enzyme activities responsible for the terminal lysis of the bdelloplast wall (E. Martin and S. C. Rittenberg, Abstr, Annu. Meet. Am. Soc. Microbiol. 1980, I108, p. 102), and the demonstration that EDTA treatment of the bdelloplast renders its modified peptidoglycan (13) accessible to exogenously added lytic enzymes (S. Suehiro and W. Cover, unpublished data), suggested that premature release of the intraperiplasmic bdellovibrio should be feasible. This would eliminate the experimental difficulty mentioned above and facilitate the study of bdellovibrio differentiation. We report here the development of an effective method for prematurely releasing intraperiplasmically growing bdellovibrios as viable growth-phase cells, and the differentiation of these cells into attack-phase forms.

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MATERIALS AND METHODS

Organisms and general culturing procedure. *B. bacteriovorus* 109J was maintained on *Escherichia coli* ML35, as previously described (13). Pregrown *E. coli* ML35 or W7-M5, a lysine and diaminopimelic acid (DAP) double auxotroph that is also unable to catabolize glucosamine (13), was used as the substrate for bdellovibrio cultures. When appropriate, peptidoglycan amino sugars and DAP residues of W7-M5 were labeled with [^{14}C]glucosamine and [^{3}H]DAP, respectively (13). Bdellovibrios (1×10^{10} to 1.5×10^{10} cells per ml) were suspended in HM buffer (1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid adjusted to pH 7.6 with NaOH, plus 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$) with *E. coli* W7-M5 (5×10^9 cells per ml) and incubated with vigorous shaking at 30°C. The resulting synchronous single cycle of growth took about 2.5 to 3 h to complete.

Media and chemicals. NB medium consisted of 0.5% nutrient broth (Difco Laboratories, Detroit, Mich.) and 0.3% yeast extract (Difco); DNB medium was prepared with 0.08% nutrient broth, 0.01% yeast extract, 0.05% Casamino Acids (Difco), and, after autoclaving, 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$. TYE medium contained 3% tryptone (Difco), 1.5% yeast extract, and 0.5% NaCl; and CY medium consisted of 0.3% Casamino Acids and 0.1% yeast extract. All media were adjusted to pH 7.6 with 1 M sodium hydroxide. [G-^{3}H]DAP, [*U*-^{14}C]leucine, and [*U*-^{14}C]UMP were obtained from Amersham Corp., Arlington Heights, Ill.; [1-^{14}C]glucosamine was obtained from New England Nuclear Corp., Boston Mass.; and [*methyl*-^{3}H]thymidine and [4,5-^{3}H]leucine were obtained from ICN Pharmaceuticals, Inc., Davis, Calif. Stock solutions of chloramphenicol (CAM [3 mg/ml]), puromycin (1 mg/ml), and novobiocin (10 mg/ml) were made fresh daily from products from Sigma Chemical Co., St. Louis, Mo. Rifamycin (stock solution as 5 mg/ml in 70% ethanol) was obtained from Calbiochem, La Jolla, Calif.

Microscopy. Phase-contrast photomicroscopy was done on a Zeiss Universal microscope. Cultures (5 μl) were absorbed on slides coated with a 2% agar film, covered with a cover slip, and photographed with Kodak Plus-X pan film (Eastman Kodak Co., Rochester, N.Y.)

Plaque assay for bdellovibrios. Bdellovibrios were enumerated by plaque assay, using the double-layer technique (15). An appropriate bdellovibrio dilution was combined with 0.2 ml of a culture of the substrate organism, *E. coli* ML35 (10^{10} cells per ml), and 2.5 ml of liquified soft agar (DNB medium containing 0.6% agar) at 42°C. The mixture was immediately spread over the surface of DNB medium containing 1.2% agar. Plaques were counted after 4 to 5 days of incubation at 30°C. Each determination is reported as the mean and standard deviation of values obtained by counting a total of 10 plates of three different dilutions.

Radioactive label incorporation and release. The kinetics of incorporation of labeled thymidine, UMP, and leucine into cold trichloroacetic acid (TCA)-insoluble macromolecules was determined as described by Kimchi and Rosenberg (7). Samples of 50 to 75 μl of culture were spotted onto filter paper (Whatman 3MM; Whatman, Inc., Clifton, N.J.) that had been saturated with 10% TCA in ether and air-dried. One hour after spotting, the sheets of paper were washed three times (15 min each) in aqueous 10% TCA (0°C). This was followed by sequential washing in 100% ethanol, ethanol-ether (1:1, vol/vol) twice, and ether. The sheets were cut into squares, which were inserted in vials containing 7 ml of a toluene-based cocktail (3 liters of toluene, 1 liter of Triton X-100, 16 g of 2,5-diphenyloxazole, and 1.4 g of 1,4-bis-2-(4-methyl,5-phenylaxazolyl)benzene for scintillation counting. Radiolabeled DAP and glucosamine residues were released from bdelloplast peptidoglycan as previously described (13). Equal volumes of culture and 10% TCA were mixed and held at 0°C for at least 1 h, and the resulting precipitate was collected by centrifugation at 20,000 × *g* for 10 min. Samples of the supernatant containing the released DAP and glucosamine were transferred to PCS solubilizer fluid (Amersham) for counting.

Chemical assays. Protein was estimated by a modified Lowry technique (9) with bovine serum albumin as the standard. Peptidoglycan (hot sodium dodecyl sulfate-EDTA-insoluble material) was isolated as previously described (13).

Preparation of LEC. Lytic enzyme concentrate (LEC) was prepared by the following procedure. Bdellovibrios (10^{10} cells per ml) were combined with tryptone-yeast extract-grown ML35 (5×10^9 cells per ml) in 300 ml of HM buffer in a 2-liter flask. Twelve such cultures (a total of 3.6 liters) were incubated at 35°C with shaking at 400 rpm. Synchronous release of progeny bdellovibrios was observed between 165 and 175 min and was accompanied by the appearance in the culture medium of lytic enzyme activities capable of solubilizing isolated bdelloplast wall peptidoglycan. Dithiothreitol (50 μg/ml) was added to the cultures, which were then cooled to 0°C. All subsequent steps were performed at or below 4°C. The culture fluid was cleared of cells and debris by two centrifugations (10,000 × *g* for 20 min each), and the resulting supernatant fluid was concentrated 60-fold by pressure filtration using a PM 30 filter (Amicon Corp., Lexington, Mass.) with a molecular size cutoff of about 30,000 daltons. The concentrate, containing all the measurable lytic activity, was further clarified by centrifugation (10,000 × *g* for 20 min), and polyethylene glycol (0.5%) was added as a stabilizing agent. Samples of the concentrate were quick-frozen in liquid nitrogen and stored at −70°C for periods of at least 2 months without appreciable loss of activity. Four batches of LEC were prepared over the course of a year. There was no significant variation observed in the effectiveness of the batches in bringing about bdellovibrio release.

Premature release procedure. *E. coli* W7-M5 was used as the substrate in studies requiring premature release, both because its peptidoglycan could be specifically labeled, and because it was somewhat more susceptible than *E. coli* ML35 to the LEC. Synchronous single-cycle cultures were allowed to progress for anywhere from 30 to 150 min of a 150-to 180-min growth cycle. At the desired time, the culture was chilled to 0°C and centrifuged at 1,100 × *g* for 5 min. The low-speed supernatant fluid, containing primarily excess bdellovibrios that had not entered or attached to prey cells, was discarded, and the loose pellet of bdelloplasts (the spherical structures consisting of substrate cells, each invaded by a bdellovibrio) was

resuspended by vigorous vortexing in HM buffer at 0°C. The centrifugation and washing were repeated twice, yielding a pellet virtually free of extracellular bdellovibrios as judged by phase-contrast microscopy. The pellet was warmed to room temperature and rapidly suspended in twice the pellet volume of 10 mM EDTA in 120 mM Tris-hydrochloride buffer (pH 7.6), and incubated for 3 min at 30°C. Ten volumes of LEC, usually containing 50 μg of CAM per ml to prevent further development, were added, and the cells were maintained at 30°C for 15 to 20 min. The suspension was cooled to 0°C, and the cells were harvested by centrifugation (10,000 × *g* for 20 min) and suspended in 1 ml of HM buffer. The released bdellovibrios were then purified by using a 25-ml linear Ficoll (type 400) gradient (2 to 15%) prepared in HM buffer and centrifuged at 140,000 × *g* for 7 min at 0°C. The released bdellovibrios formed a visible band midway down the gradient, well above the pellet containing unlysed bdelloplasts (typically less than 10% of the total initial number) and cell debris. The band was removed to a centrifuge tube, and after three washings in HM buffer (0°C), the released bdellovibrios were used for subsequent study.

RESULTS

Premature release of intraperiplasmic bdellovibrios. The sequence and results of applying the premature release technique to a 90-min synchronous bdellovibrio culture are summarized in Fig. 1. Attack-phase bdellovibrios and prey cells were combined at about a 3:1 ratio (Fig. 1a) to achieve optimum synchronization (Fig. 1, step 1). After penetration and formation of the bdelloplasts, the intraperiplasmic bdellovibrios began to grow (Fig. 1, step 2). Excess nonadherent and nonpenetrating bdellovibrios were removed by repeated low-speed centrifugations (Fig. 1, step 3). Greater than 95% of the excess bdellovibrios remained in the discarded supernatant fluid, whereas about 50% of the quickly sedimenting bdelloplasts were recovered in the final pellet. This procedure produced a relatively pure preparation of bdelloplasts essentially free of contaminating attack-phase bdellovibrios. (Fig. 1b).

Treatment of these bdelloplasts with EDTA (Fig. 1, step 4) increased the permeability of the bdelloplast envelopes to the entry or exit of macromolecules. Within a few minutes of treatment, substrate-cell cytoplasmic proteins were released into the culture fluid (W. Cover, unpublished data), and the bdelloplasts became transparent (Fig. 1c). The bdellovibrio filaments within the bdelloplasts remained opaque and were not harmed by the brief exposure to the EDTA concentration used as was indicated by their continued high viability, their normal rate of protein synthesis after treatment, and their ability to fragment and differentiate (see below).

When the LEC was added to the permeabilized bdelloplast suspension, the bdelloplast walls were solubilized and the growth-phase bdellovibrios were released (Fig. 1, step 5). The released cells were separated from residual bdelloplasts and cellular debris on a Ficoll density gradient (Fig. 1, step 6), yielding a pure prepara-

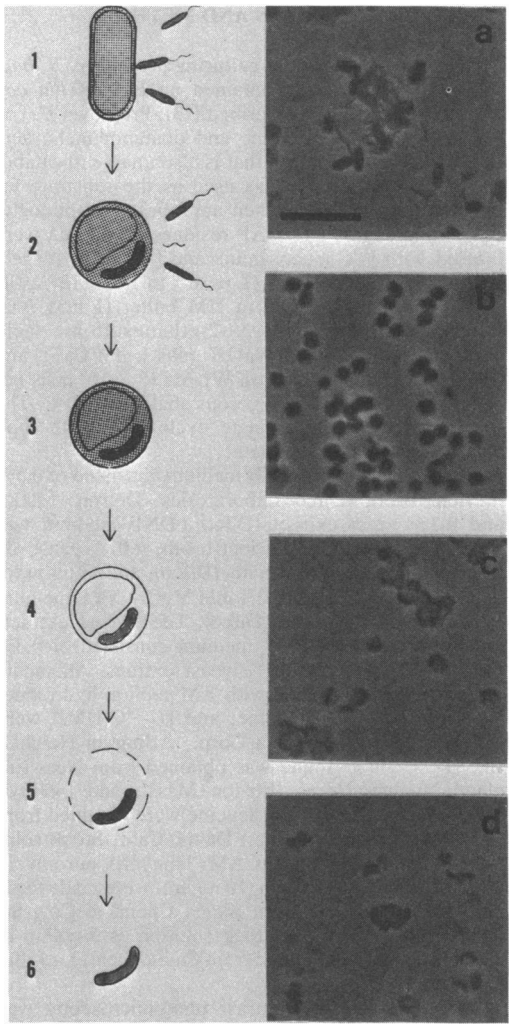

FIG. 1. Steps 1 to 6, Flow diagram summarizing the procedure for prematurely releasing intraperiplasmic bdellovibrios. Step 1, Substrate cells were synchronously attacked by bdellovibrios; step 2, penetrated substrate cells were converted to bdelloplasts and bdellovibrios began intraperiplasmic growth; step 3, excess bdellovibrios were removed by differential centrifugation; step 4, bdelloplasts were briefly treated with EDTA and became transparent; step 5, bdelloplast envelopes were lysed by LEC, releasing the bdellovibrios; step 6, prematurely released bdellovibrios purified by Ficoll gradient centrifugation. (a to d) Photomicrographs of a synchronous culture at steps 1, 3, 4, and 6 in the procedure; bdelloplasts processed at 90 min. Bar, 10 μm.

TABLE 1. Solubilization of bdelloplast peptidoglycan by treatment with EDTA or LEC or both[a]

Treatment	% Labeled peptidoglycan solubilized[b]	
	[^{3}H]DAP	[^{14}C]glucosamine
Buffer	0	0
EDTA	0	15
LEC	7	0
EDTA followed by LEC	80	76

[a] A 60-min synchronous culture of *B. bacteriovorous* 109J growing on [^{3}H]DAP-, [^{14}C]glucosamine-labeled *E. coli* W7-M5 was processed through step 3 (Fig. 1) as described. Portions of the resulting radioactive bdelloplast suspension (^{14}C, 21,000 cpm/ml; ^{3}H, 8,000 cpm/ml) were incubated at 30°C in HM buffer (2 min), EDTA (2 min), LEC (30 min), or EDTA (2 min) then LEC (20 min). Hot sodium dodecyl sulfate-insoluble material was isolated from the treated suspensions, and the radioactivities were determined.

[b] Percent decrease in label appearing in hot sodium dodecyl sulfate-insoluble material after treatment.

tion of viable metabolically active growth-phase bdellovibrios (Fig. 1d). Viability approaching 100% of the released cells was determined by comparisons of plaque counts with direct counts made by using epifluorescence microscopy.

The growth-phase bdellovibrios released from 90-min bdelloplasts (Fig. 1d) appeared thicker, longer, and more curved than the attack-phase cells used to initiate the culture (Fig. 1a). As previously reported (11), attack-phase cells of *B. bacteriovorus* 109J are small comma-shaped vibrios released from the bdelloplast at the end of normal intraperiplasmic growth, but they elongate and straighten with time, reaching the morphology seen in Fig. 1a. Bdellovibrios prematurely released from *E. coli* bdelloplasts late in the growth phase were coiled nonseptate filaments up to about 4 cell lengths long, whereas those prematurely released from large substrate cells, such as *Spirillum serpens*, could be very long filaments of 10 or more cell lengths.

Serial treatment of bdelloplasts with EDTA and the LEC solubilized both DAP and glucosamine residues of the bdelloplast peptidoglycan as indicated by the loss of both ^{3}H and ^{14}C radioactivity from the residual hot sodium dodecyl sulfate-insoluble material (Table 1). The pretreatment of bdelloplasts with EDTA was necessary for an effective digestion of peptidoglycan by the LEC, further illustrating the role of EDTA in making the bdelloplast envelopes permeable to macromolecules. It should be noted that after EDTA treatment the addition of 10 volumes of LEC (in HM buffer) rendered insignificant any residual chelating activity of the EDTA. This dilution was necessary because the lytic enzyme activity requires the presence of free divalent ions (E. Martin, unpublished data).

Because the two labels were lost in the same proportion, either could be used to monitor changes in total peptidoglycan. The kinetics of solubilization of [^{3}H]DAP showed that about 80% of the bdelloplast wall peptidoglycan was released into the culture fluid within 20 min (Fig. 2). The rate of release was unaffected by the presence of CAM (50 μg/ml) or potassium cyanide (3 mM), but was essentially eliminated when the LEC was heat-treated (90°C, 1 min) before use. Thus, the enzymatic activities of the concentrate were responsible for the solubilization of the bdelloplast walls and release of the bdellovibrios, whereas metabolic activity by the bdellovibrios themselves was neither responsible nor required.

The premature-release technique was also applied successfully to bdellovibrios growing intraperiplasmically in other prey strains (*E. coli* ML35, *Pseudomonas putida* N-16, and *Spirillum serpens* VHL), although not necessarily with the same effectiveness.

Differentiation of released bdellovibrios. Bdellovibrios, released at any time during their 2.5- to 3-h intraperiplasmic growth phase, differentiated, upon further incubation, from nonflagellated, plump growth-phase cells into flagellat-

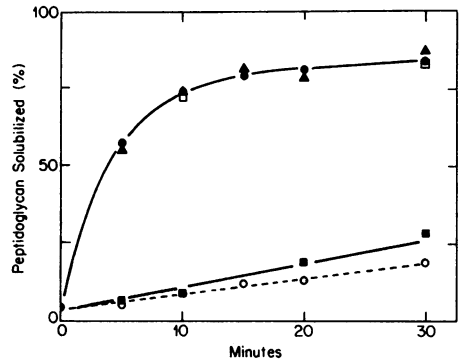

FIG. 2. Solubilization of the peptidoglycan of EDTA-treated bdelloplasts by the LEC. A 60-min synchronous culture of bdellovibrios growing on [^{3}H]DAP-labeled *E. coli* W7-M5 was processed as given in the legend to Fig. 1 through step 4. The treated bdelloplasts were diluted into HM buffer (○), LEC (●), LEC plus 50 μg of CAM per ml (▲), LEC plus 3 mM KCN (□), or heated LEC (90°C, 1 min) (■). Suspensions were shaken at 30°C and sampled as indicated, and the radioactivity of cold 5% TCA-soluble fractions of the samples was determined. Percent peptidoglycan solubilized is presented as: (TCA-soluble radioactivity/ total radioactivity) × 100%. Total counts of samples (100%) ranged from 10,000 to 14,000 cpm/ml. These results are typical of several such determinations.

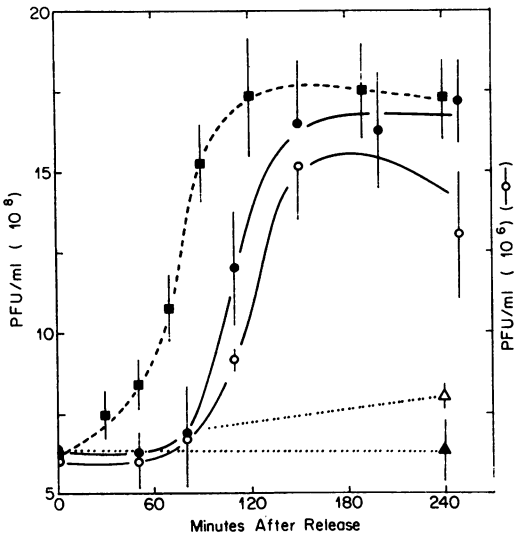

FIG. 3. Kinetics of fragmentation of prematurely released growth-phase bdellovibrios in CY medium and HM buffer and at different culture densities. At 125 min into the growth cycle, intraperiplasmic bdellovibrios were released, washed, suspended either in HM buffer at 6×10^8 (●) or 6×10^6 (○) cells per ml or in CY medium at 6×10^8 cells per ml (■), and incubated at 30°C. At 0 min (▲) and 80 min (△) after release, CAM (100 μg/ml) was added to a portion of the 125-min culture, and the incubation was continued. Periodically, the number of viable progeny cells (PFU per ml) was determined by plaque assay of diluted samples. Error bars cover ± one standard deviation for replicate samples in a single typical experiment.

ed, motile thin rods characteristic of normally releasd attack-phase cells. During incubation, motile cells were observed by about 30 min, and essentially all cells of the culture were motile by 90 min after premature release. In the presence of prey bacteria, the differentiated cells were fully capable of initiating subsequent intraperiplasmic growth cycles. The differentiation process occurred not only in media, but also when well-washed released cells were incubated in the nonnutrient HM buffer. Differentiation required both oxygen, for respiration, and divalent cations. The addition of EDTA reversibly halted the transformation. Differentiation was also inhibited by CAM (50 μg/ml), puromycin (500 μg/ml), and rifamycin (200 μg/ml).

When sufficient time in the intraperiplasmic environment had elapsed before release, differentiation also involved fragmentation of elongated cells, as evidenced by an increase in PFU during postrelease incubation. An increase in numbers was detectable as early as 30 min after release for cells suspended in CY medium and later, at about 75 min, for cells in HM buffer (Fig. 3). The time of initiation of fragmentation in buffer was independent of cell concentration. Fragmentation, once begun, proceeded at similar rates in CY medium and HM buffer. Chloramphenicol, added either at the time of release or after initiation of differentiation, inhibited fragmentation.

Although the rate of fragmentation was similar in cells released anytime between 75 and 120 min into the intraperiplasmic growth cycle, the number of cells produced by the fragmentation process was dependent upon the time of release. Cells released at 60 min or less into the cycle did not fragment, and the number of viable attack-phase progeny cells produced equaled the number of growth-phase cells released (Fig. 4). Cells

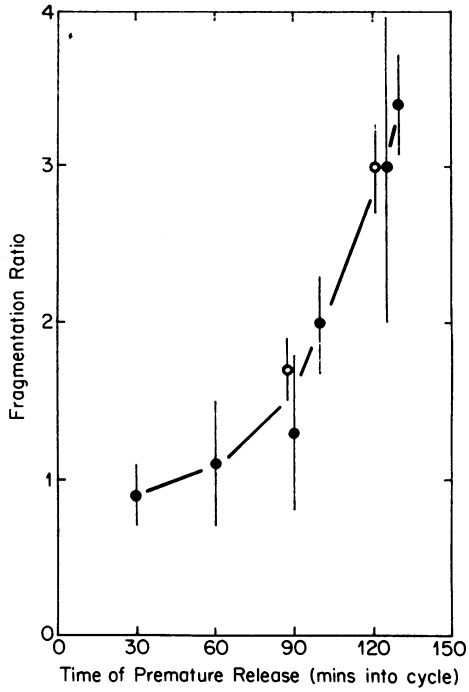

FIG. 4. Relationship between the time of release and the number of progeny cells produced per released cell. At the indicated intervals into the 160-min growth cycle, intraperiplasmic bdellovibrios were released, washed, suspended in HM buffer (●) or in CY medium (○) to between 2×10^9 and 5×10^9 cells per ml, and incubated at 30°C for an additional 300 min. The number of viable cells was determined on diluted samples of the initial and final cultures. The plaque count at 300 min divided by the initial plaque count, i.e., the fragmentation ratio, was plotted as a function of the age of the bdellovibrio filament at the time of premature release. For clarity, the CY medium points are displaced a short distance to the left of the corresponding HM buffer points. Error bars cover ± one standard deviation. Normal completion of the 160-min growth cycle yielded an average of four progeny cells per bdelloplast.

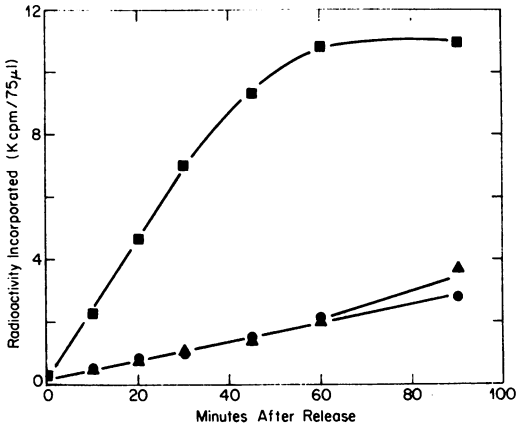

FIG. 5. Syntheses of DNA, RNA, and protein by prematurely released growth-phase bdellovibrios during differentiation. At 75 min into the growth cycle, intraperiplasmic bdellovibrios were released, washed, and suspended to 5×10^8 cells per ml in CY medium containing labeled precursors (1×10^6 to 8×10^6 cpm per ml), and the cultures were incubated at 30°C. At intervals, 75-μl samples were removed for determination of [^{3}H]thymidine (■), [^{14}C]UMP (▲), or [^{3}H]leucine (●) incorporation into cold TCA-insoluble material.

released after 60 min subsequently underwent fragmentation, and, as premature release was delayed later and later into the growth phase, the number of viable attack-phase progeny produced per released cell approached four, the yield in uninterrupted cultures. The yield was not significantly different between released cells incubated in buffer or in CY or NB medium.

Bdellovibrios, prematurely released at any time in their intraperiplasmic growth cycle, synthesized protein and RNA at approximately a linear rate for at least 3 or 4 h when suspended in CY medium (Fig. 5). DNA synthesis, as measured by thymidine incorporation, showed a different pattern. Cells released before 50 min of intraperiplasmic growth incorporated little or no thymidine during subsequent differentiation in CY medium (Fig. 6), whereas significant incorporation occurred in cells released from 50 min on. Clearly, the initial rates of DNA synthesis per cell as well as the extent of incorporation increased with increasing time of release of the cells. In no case, however, did thymidine incorporation continue longer than 90 to 100 min after release, although incorporation of protein and RNA precursors continued well beyond this time at an essentially undiminished rate.

Thymidine incorporation by released cells could be specifically and reversibly inhibited by novobiocin, a DNA gyrase inhibitor (2). The addition of novobiocin reversibly suspended differentiation by DNA-synthesizing released cells, but had no effect if added after thymidine incorporation had ceased (i.e., 100 min post release). Initial addition of novobiocin (100 μg/ml) to released bdellovibrios decreased their rate of thymidine incorporation by a factor of 10 (Fig. 7). Removal of the inhibitor by washing led to an almost immediate increase in incorporation at a rate approximating that initially exhibited by the control culture. Regardless of the time of removal of inhibitor, uptake continued until approximately the same total amount of thymidine had been incorporated.

DISCUSSION

We describe here a method for the premature release of bdellovibrios from their intraperiplasmic growth environment. This technique provides a virtually 100% viable population of synchronous growth-phase cells uncontaminated with attack-phase cells or debris. The released bdellovibrios are obtained quickly with a minimum opportunity for metabolic adjustment to their new surroundings. The entire process, beginning with the purified bdelloplasts, takes less than 60 min, during most of which time the cells are held at 0°C. Additionally, the incubation in LEC for 20 min at 30°C can be performed in the presence of CAM, which reversibly inhibits the onset of differentiation.

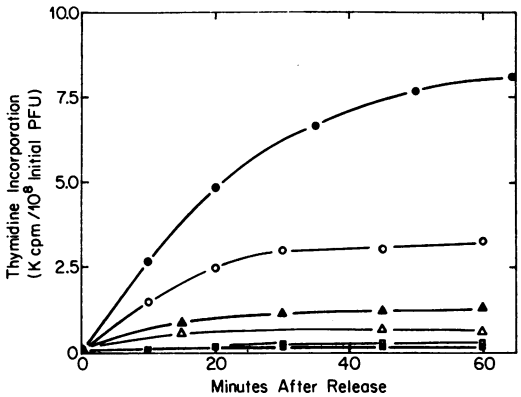

FIG. 6. Rates of DNA synthesis by growth-phase bdellovibrios released at different times in the growth cycle. At 35 (■), 40 (□), 50 (△), 60 (▲), 75 (○), and 100 (●) min into the growth cycle, intraperiplasmic bdellovibrios were released, washed, and suspended to about 3×10^9 cells per ml in CY medium containing [^{3}H]thymidine, and the cultures were incubated at 30°C. At intervals, 50-μl samples were removed for determination of label incorporation into cold TCA-insoluble material. Rates were normalized to the initial number of viable released growth-form cells as determined by plaque assay of diluted samples.

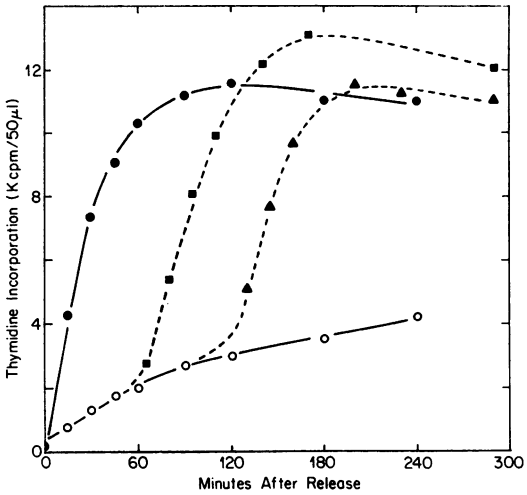

FIG. 7. Rates of DNA synthesis of growth-phase bdellovibrios during and after novobiocin inhibition. At 125 min into the growth cycle, intraperiplasmic bdellovibrios were released, washed, and suspended to 5 × 10^9 cells per ml in CY medium containing [^{3}H]thymidine, with (○) or without (●) 100 µg of novobiocin per ml, and the cultures were incubated at 30°C. At intervals, 50-µl samples were removed for determination of label incorporation into cold TCA-insoluble material. After an additional 50 (■) and 100 (▲) min, a portion of the culture containing novobiocin was washed twice with HM buffer and suspended in CY medium without novobiocin.

The synchronous released cells were used to examine a critical developmental event in their unique dimorphic growth cycle: the differentiation from growth-phase filaments to motile attack-phase progeny cells. Growth-phase bdellovibrios exhibited considerable adaptability, being capable of differentiating at any time and totally at the expense of their own cellular reserves: no external carbon or energy source was needed and no extracellular effectors were required. Suspension of growth-phase cells in a nutrient medium neither prolonged the growth phase nor delayed the beginning of differentiation, even though the medium was nutritionally sufficient for growth of axenic mutants (K. Nishimoto, unpublished data). Thus, the prematurely released growth-phase cells of obligately predatory bdellovibrios still retain the inability to grow in nutrient medium, the trait that distinguishes them from axenic strains (14). In fact, differentiation of released cells, as judged by the onset of fragmentation, began sooner in growth-phase cells incubated in nutrient medium than those incubated in buffer (Fig. 3). The data given above indicate that differentiation is a direct consequence of its being removed from some positive chemical or physical condition of the intraperiplasmic environment. Furthermore, they suggest that in the normal intraperiplasmic cycle, differentiation is initiated by the exhaustion of a regulatory compound(s), but that once initiated, it is accelerated by a compliment of nutrients.

Two aspects of differentiation that were specifically investigated were; the fragmentation of prematurely released cells into multiple progeny cells, and DNA synthesis by cells after release. Not surprisingly, the data indicate a direct relationship between these two events.

When sufficient time in the intraperiplasmic environment elapsed before release, at least some of the released cells fragmented during the differentiation process. For this to occur, the cells must have had a minimum period of intraperiplasmic existence of between about 45 and 60 min after attack. This corresponds to the time at which DNA synthesis by intraperiplasmic bdellovibrios appears to be initiated (8). Fragmentation was directly related to filament length at the time of release and probably to the number of completed or initiated genomes (see below).

Because the kinetics of fragmentation are similar for released growth-phase bdellovibrios suspended at two different cell densities (Fig. 3), it is unlikely that initiation of fragmentation depends upon the extracellular accumulation of a "division factor" as has been reported for axenically growing bdellovibrio (3) and a filamentous cyanobacterium (6). This observation does not rule out the possibility that some factor may have to accumulate intracellularly.

Attack-phase bdellovibrios do not incorporate thymidine (10), nor have we found that they synthesize DNA when incubated in nutrient medium. Thus, it is their entry, or some event subsequent to entry into the prey cell, that presumably signals initiation of chromosome replication. Studies of intraperiplasmically growing cells (8) have shown that, although entry into and structural modification of the prey cell occur 20 min after attack, DNA synthesis cannot be detected until about 45 to 60 min. These data (8) can be explained in two ways: upon entry, the bdellovibrios receive a signal that commits them to initiate DNA synthesis, but an additional 40 min is required to implement the signal; or, the bdellovibrios must exist intraperiplasmically for about 60 min before becoming committed to DNA synthesis. The latter explanation is supported by the observations made here that only those growth-phase cells released after 50 min synthesize DNA and subsequently fragment into progeny cells. Before this time, intraperiplasmically growing cells apparently have not become committed to chromosome replication.

The kinetics of postrelease DNA synthesis suggest the completion of already initiated rounds of replication, but not initiation of new rounds. First, there is a similarity between the reported time at which intraperiplasmic DNA synthesis begins (8) and the minimum release time after which the cells are competent for postrelease DNA synthesis (Fig. 6). Second, there is a proportionality between the apparent initial rate and the extent of synthesis (Fig. 6). Assuming that the specific rate of DNA synthesis is constant (4), then the final amount made is a direct function of the number of chromosome replications underway at the time of release. Subsequent initiations, were they to occur, would be expected to eliminate this proportionality. Third, novobiocin inhibits the rate of thymidine incorporation by released cells, but the decreased rate of incorporation continues past the time that uninhibited cultures cease synthesis. In addition, cells removed from inhibition at any time go on to incorporate about the same amount of thymidine as control cells. Both of these observations indicate that it is the amount of DNA to be synthesized, and not the extent of time after release, that determines how long DNA synthesis will continue. The inhibitory effect of novobiocin on differentiation further suggests that termination of DNA synthesis is required for completion of the transformation of growth-phase cells into attack-phase bdellovibrios. Cells treated with novobiocin before they complete DNA synthesis do not go on to differentiate, whereas the addition of the drug to cells after synthesis is complete does not affect subsequent differentiation.

This work relates directly to a fundamental question in the study of bdellovibrios: the nature of the prey dependency. A strong argument can now be made that this dependency is regulatory and not nutritional. Not only are prematurely released growth-phase bdellovibrios capable of a morphological and physiological transformation into attack-phase cells without external nutrients, but they can also complete already initiated rounds of chromosome replication under the same conditions. Thus, the potential to synthesize DNA is independent of any special nutrient in the environment. Because postrelease DNA synthesis apparently involves only termination of intraperiplasmically initiated rounds, each round of replication by intraperiplasmically growing bdellovibrios must require a new initiation signal. Thus, not only is the initiation of DNA synthesis a regulatory event controlling the onset of intraperiplasmic growth, as previously hypothesized (3, 15), but also the initiation of subsequent rounds continues to be regulated by some signal associated with the intraperiplasmic environment.

It is important to note that another level of regulation is apparent from the obligatory coupling of cell differentiation and DNA synthesis. Axenic strains can only multiply in nutrient medium after differentiation into the unflagellated, relatively plump growth-phase cell morphology and grow as the characteristic coiled filament that elongates until the nutrients are depleted and the cell fragments into motile, nongrowing attack-phase progeny (1, 5). However, differentiated (growth-phase) prey-dependent bdellovibrios, if suspended in nutrient medium, will not continue to multiply. Thus, both the growth-phase morphology as well as a signal to initiate DNA synthesis are required for multiplication outside of the prey cell. Conversely, differentiation (including fragmentation) from the growth-phase morphology to the attack-phase morphology is similarly coupled to completion of any round of DNA synthesis interrupted by premature release, even though the released growth-phase filament may contain several presumably completed genome copies.

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