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An investigation of some of the physiological properties of Bdellovibrio bacteriovorus 109D was undertaken. This investigation was divided into two phases. In the first phase of the study, a variety of temperature-sensitive mutants of host-dependent Bdellovibrio bacteriovorus 109D were selected after ethyl methane sulfonate mutagenesis. Mutants that demonstrated plaque-forming ability reversion frequencies of 10^{-8} to 10^{-9} were chosen for further study. Representatives of these mutants were then characterized by phase-contrast and electron microscopy, temperature-shifted one-step growth experiments, attachment kinetics, and macromolecular capabilities. Representative mutants demonstrate various types of blockage corresponding to the previously described morphological stages of Bdellovibrio predatious life cycle, i.e., attachment blockage (109D153), penetration blockage (109D3 and 109D48), and blockage of intracellular growth (109D4 and 109D152). The time of release from temperature repression for the mutant classes was found to correspond to the apparent morpholgical stage of blockage via temperature-

shifted, one-step growth experiments. Mutants characterized as exhibiting blockage in the penetration or intracellular stages of the infection cycle exhibited, at the permissive and nonpermissive temperatures, kinetics of attachment to Escherichia coli WP2 similar to those of the wild-type. One mutant, 109D153, exhibited depressed attachment at the restrictive temperature even though the Bdellovibrio cells were motile. The extent of 38.5C attachment of 109D153 to E. coli is at the same level as that of wild-type 109D to Bacillus subtilis, a gram-positive, non-host organism. Subsequent detachments were revealed in the wild-type 109D-B. subtilis or mutant 109D153-Escherichia coli (38.5C) cultures. These studies reveal a biphasic attachment phenomenon in the early interaction of Bdellovibrio with its host. It appears that, at the restrictive temperature, 109D153 is capable only of the initial, nonspecific type of attachment.

In the second phase of this investigation, the initiation and kinetics of Bdellovibrio bacteriovorus 109D deoxyribonucleic acid synthesis following the infection of a suitable host were monitored via density shift experiments. When 5-bromouracil substituted E. coli UC12 cells were infected with Bdellovibrio bacteriovorus 109D cells containing DNA isotopically labeled with 6-^{3}H-thymidine, cesium chloride equilibrium density gradient centrifugation revealed the formation of an isotopically labeled light/heavy hybrid DNA peak. This density-shifted, isotopically labeled peak was not present immediately after infection, nor was it present 65 minutes into the

infection cycle. Its presence was first noted 75 minutes after the initiation of infection. By 85 minutes after infection this peak had reached a maximum level, which was apparently maintained thereafter. When 6[^{3}H]5-bromouracil substituted E. coli UC12 cells were infected under similar conditions, cesium chloride equilibrium density gradient centrifugation revealed a progressive loss of counts in the lower regions of the gradients and the emergence of labeled molecules in the upper portions of the gradients. Although some of these counts were released to heavier density positions following shearing, in later time samples an increasing proportion were not. These observations are interpreted as indicating a major incorporation of density label into Bdellovibrio deoxyribonucleic acid in a random manner.

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I. INTRODUCTION

In 1962 Heinz Stolp (79) observed the formation of plaques which were delayed in onset and which continued to spread upon prolonged incubation. Bacteriophage plaque formation on lawns of sensitive bacteria is generally rapid in onset, each replication cycle being completed in less than an hour and liberating hundreds of progeny. Moreover, bacteriophage plaque size is generally limited; viral replication halts when energy dependent systems of the bacterial lawn are shut down at the end of a short, active growth period. Stolp's recognition of the unusual behavior of this plaque type led to his discovery of a new bacterium.

When Stolp examined the new plaques, encountered following the inoculation of soil filtrate onto *Pseudomonas phaseolicola*, he observed minute bacteria which collided with, attached to, and caused the lysis of the host pseudomonad cells. The apparent parasitic nature and small size of this bacterium later led Stolp and Starr (89) to propose a new genus, *Bdellovibrio*, and species, *bacteriovorus*, after examining a total of eleven soil and sewage isolates. This genus has now been subdivided into three species (54) and has been the subject of numerous reviews (61, 68, 69).

Bdellovibrio have now been isolated from soil, sewage, fresh water, and marine sources (26, 37, 69). Biological densities as high as 70,000 cells/g of soil

and 100,000 cells/ml of sewage have been reported (37, 69). The cell densities from aqueous sources reported are low, though localized concentrations undoubtedly occur (46).

Several authors have worked toward a comprehensive definition of the genus. As set forward by Starr and Seidler (69) Bdellovibrio is:

> "the genus of bacterial prokaryotes which are: (a) generally vibrioid, sometimes spirillar; (b) unusually narrow (about 0.3-0.45 μm in width); (c) polarly flagellated (very rarely with more than one flagellum but the flagellum is always sheathed and the insertion is always polar); (d) gram-negative; (e) strictly aerobic; (f) chemo-organotrophic; (g) endowed with DNA which is highly homogeneous and contains 42-51 moles per-cent G+C; (h) markedly and invariably proteolytic; (i) able to metabolize through the TCA cycle; (j) generally not capable of using carbohydrates; (k) fitted with a dimorphic development cycle: alter-nation of flagellated, predatory, vibrioid swarmers (capable of attaching by the aflagellated tip to a host cell) and nonflagellated spirillar (usually intracellular) vegetative stages; and, most importantly, (l) actually capable of (or have the genetic potential for) entering the cells of certain bacteria and developing and multiplying therein."

The dependence on another bacterium for normal growth and the competence to establish and maintain a specific association with it are two unique properties of Bdellovibrio. As early as 1963, however, mutants of Bdellovibrio were isolated which were capable of axenic growth in complex medium. These mutants, isolated at a frequency of approximately one in 10^6 cells, were termed saprophytic or host-independent. Later, certain Bdellovibrio strains were recognized as having characteristics intermediate between those of the wild-type

host-dependent and the isolated host-independent strains. These isolates were described as being facultative in nature. Varon and Seijffers (86) have suggested the following nomenclature for the three classes of Bdellovibrio mutants which have been described:

> "i) $S^{in}comp^{+}$ for those that have been described as being facultative (63, 86) and that can establish a symbiotic relationship with other bacteria but do not depend on them for normal growth; (ii) $S^{in}comp^{-}$ for mutants that have been described as being nonparasitic (85) or nonsymbiotic (66) and that have lost their symbiotic competence but gained the capacity for independent growth; (iii) $S^{d}comp^{-}$ for mutants that are conditional and that are competent at the permissive temperature but incompetent at higher, restrictive temperatures (20, 62)."

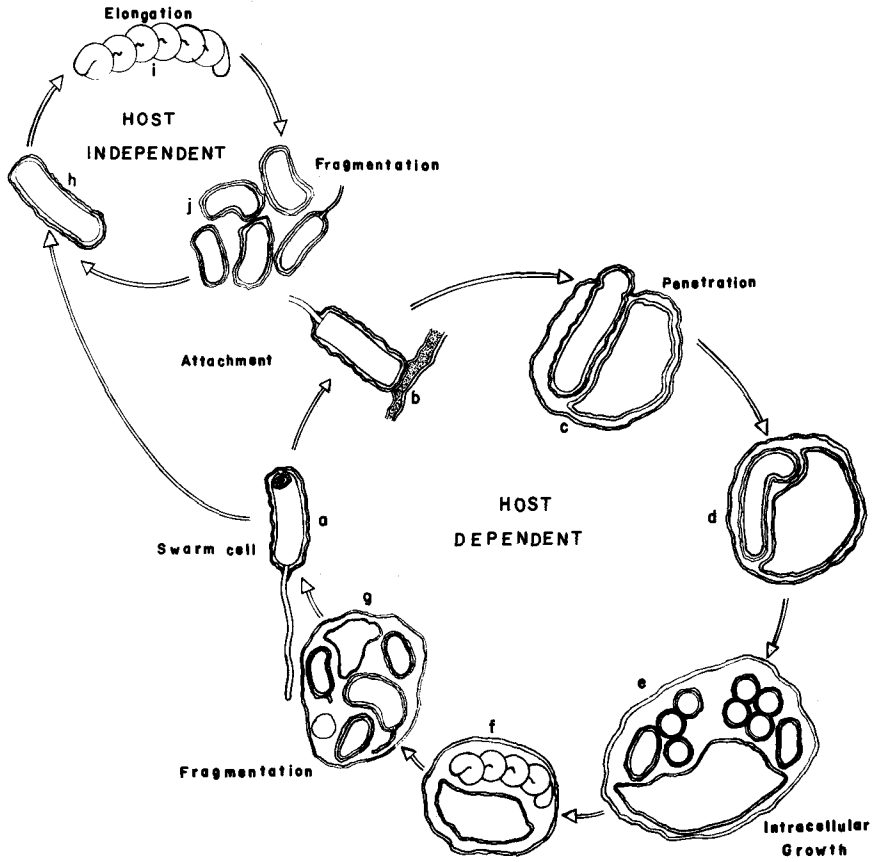
It should be noted here that, as pointed out by Starr and Seidler (69), facultative parasitism is always a transient characteristic for which there is no positive selection upon repeated growth in host-free media.

LIFE CYCLE

In culture, Bdellovibrio may be observed to undergo the dimorphic developmental cycle illustrated in Figure 1. In this cycle, a small vibrioid form, which for the sake of reference will be termed a swarm cell, differentiates to yield a replicating spiral form. There are apparently two routes available for replication: (A) host-dependent mode and (B) host-independent mode. For discussion purposes, the host-dependent replication mode may be subdivided into

Figure 1. The Bdellovibrio life cycle.

The dimorphic developmental cycle of Bdellovibrio bacteriovorus is illustrated. An actively motile swarm cell (a) attaches to (b) and penetrates (c, d) a gram negative bacterial cell and subsequently differentiates to yield a multinucleate, spiral-shaped form (e, f). This long spiral is fragmented following multiple transverse fission (g) to yield progeny swarm cells which escape the now ghosted bacterial cell and attempt to repeat the cycle. Under laboratory conditions Bdellovibrio mutants (h) may be isolated which will undergo a similar elongation (i) and division (j) on complex medium in the absence of intact host cells.


Elongation
i
HOST
INDEPENDENT
Fragmentation
h
j
Attachment
b
Penetration
c
d
HOST
DEPENDENT
Swarm cell
a
g
Fragmentation
f
e
Intracellular
Growth

four phases, each of which is characterized by an observed developmental event:

I) Free-swimming Phase (Figure 1a)

II) Attachment Phase (Figure 1b)

III) Penetration Phase (Figure 1c, d)

IV) Growth and Replication Phase (Figure 1d-g)

A) Host-dependent Replication

I) Free-swimming Phase (Figure 1a)

Free-swimming Bdellovibrio swarm cells are small (0.3-0.45 μm wide and of variable length) gram-negative vibrioids. They are initially highly motile, traveling at speeds greater than 100 μm/sec which, size for size, easily qualifies them as one of the fastest biological entities known. They are propelled at these speeds by a single, polarly situated flagellum which at 28 nm in width is approximately twice the thickness of a typical bacterial flagellum. Seidler and Starr (55), by electron microscopic observations following treatment with 6 M urea, first demonstrated that this flagellum is sheathed by an extension of the Bdellovibrio cell wall.

In this motile swarm cell state, Bdellovibrio cells exhaust their intracellular energy reserves at a tremendous rate (31). This high endogenous respiration is driven by the degradation of cell protein and RNA. Ribosomal profiles show a rapid degradation of ribosomes. Concurrently a large proportion of cell protein is rapidly degraded. Hespell et al. (31) have calculated that up to 40% of this respiration may be expended upon cell motility. Under these conditions of

self-destructive respiration, Bdellovibrio swarm cells rapidly enter an immobile state which appears as a period of low energy turnover preceding cell death. The respiratory quotient remains the same over this transition, suggesting that the nature of the respired compounds does not change. The addition of complex media or specific metabolites has been demonstrated to have a sparing effect upon this respiratory death.

Bdellovibrio swarm cells are capable of only limited macromolecular synthesis. They can regenerate a lost flagellum and synthesize ribosomal RNA; however, other macromolecular capabilities apparently are in a repressed state. Neither cell replication nor DNA synthesis can occur in complex medium lacking host cell components, although cell elongation often takes place.

It has been suggested that Bdellovibrio swarm cells possess chemotactic mechanisms for the detection of host cells (87). Chemotactic attraction of Bdellovibrio swarm cells to various concentrations of yeast extract has been demonstrated by Straley and Conti (82).

II) Attachment Phase (Figure 1b)

In two-membered cultures comprised of Bdellovibrio cells and cells of a suitable host bacterium, the first stage of the Bdellovibrio host-dependent replication cycle begins when a motile swarm cell collides with and attaches to a host cell. The force of this collision is readily evident; a tiny Bdellovibrio cell often rapidly drives a host cell of 10-20 times as great a mass through the suspending medium. This observation would also seem to testify

to the firmness of the bonding involved in attachment.

The factors involved in the attachment of Bdellovibrio to a suitable host are poorly understood. Host viability is not a requirement for attachment. Varon and Shilo (87) were able to quantitate Bdellovibrio cell attachment in two-membered cultures following filtration through a 1.2 μm membrane filter. Host cells (in this case Escherichia coli) were efficiently retained while greater than 90% of the unattached Bdellovibrio cells were refractile to the trapping effect of the membrane. Using this technique, they reported that the kinetics of attachment and the final number of Bdellovibrio cells attached were dependent on the multiplicity of the parasite, the composition and pH of the medium, and the incubation temperature. Inhibitors of attachment also inhibited Bdellovibrio motility. However, concentrations of ethylenediaminetetraacetate (EDTA) which do not prevent Bdellovibrio motility have been observed to prevent Bdellovibrio attachment (87). Streptomycin, chloramphenicol, puromycin and penicillin did not affect the rate of Bdellovibrio attachment to E. coli under conditions which did lead to inhibition of Bdellovibrio host penetration by the first three antibiotics. The degree of culture aeration has been shown to affect the kinetics of Bdellovibrio attachment (87).

Using various cell envelope mutants of E. coli and Salmonella spp., Varon and Shilo (88) demonstrated an elevation of the kinetics of Bdellovibrio attachment to cells lacking the o-specific side chain. R-antigen mutant strains demonstrated a reduced rate of

Bdellovibrio attachment, possibly implicating their involvement in Bdellovibrio attachment. However, the efficiency of Bdellovibrio plaque formation on lawns of those mutant hosts tested did not vary, indicating that attachment kinetics is only of limited aid as a monitor of attachment.

Specialized structures at the anterior end of Bdellovibrio cells have been observed (2, 8, 67). It has been proposed that some of these modifications represent artifacts produced during sample preparation (2). Fine fibers (approximately 50 Å in diameter and up to 0.8 μm long) have been observed to emerge from ring-like structures associated with the Bdellovibrio cell wall and membrane. They have been postulated as playing a role in Bdellovibrio attachment to or penetration of the host cell (2) since few unattached Bdellovibrio cells demonstrate such fibers.

Bdellovibrio cells become aflagellate shortly after attachment. Bdellovibrio penetration of the host cell follows.

III) Penetration phase (Figure 1c, d)

Having encountered a suitable host cell and successfully mediated attachment to it, Bdellovibrio cells are observed to breach the host cell wall and enter the host cell periplasmic compartment. Several workers have commented on Bdellovibrio movement following attachment and have postulated on involvement of this motion in the initial lesion of the host cell leading to Bdellovibrio penetration (64, 67, 73, 74, 80). Local damage to the host cell surface has been observed to occur even though the infecting

Bdellovibrio cell may have subsequently detached (3, 60). Ultrastructural observations of two-membered cultures suggest an erosion of the host murein layer (67) and an early localized bulging of the host cell wall near the site of attachment (8, 9, 33, 40). As noted earlier, inhibitors of protein synthesis prevent successful Bdellovibrio penetration of the host cell. These observations would seem to suggest an induction of enzymatic factors aiding in Bdellovibrio entry (84). Prominent mesosomes at the anterior end of the attaching Bdellovibrio cell have been suggested as playing a role in this process (8, 10, 33, 67).

A variety of enzymes induced during host-dependent replication have been recognized. Thus, lysozyme-like muramidases (18, 34, 35), proteases (12, 14, 15, 20, 35, 54, 63, 71, 80), and a lipase (35) have been described. All these enzymes undoubtedly play an important role in Bdellovibrio replication. However, the specific role of any of these enzymes has not been demonstrated. In particular, the production of a specific enzyme has not been linked to a specific morphological event, such as host penetration.

Bdellovibrio entry of the host cell occurs through a penetration pore which is smaller than the diameter of the Bdellovibrio cell (8, 9, 67). Thus, constriction of the entering Bdellovibrio cell is observed as penetration proceeds (8). One laboratory has reported that after formation of a passage pore in the host cell wall, the Bdellovibrio penetration pore is associated with the host protoplast (1). This contact is viewed as being firm and lasting, thus leading

to a proposed penetration mechanism in which the Bdellovibrio cell is passively drawn in through the action of forces generated by fluxes of water and solutes due to structural changes in the infected host envelope.

IV) Growth and Replication Phase (Figure 1d-g)

By the time Bdellovibrio penetration is complete, the host cell has generally lost rigidity and appears as a spheroplast. This form has been termed a Bdelloplast by one laboratory (68) since the true nature of the biochemical events leading to the observed morphological change is not known.

It is in this rounded cell structure that the infecting Bdellovibrio cell, lying between the host membrane and outer envelope layers, replicates. This replication is readily observed to take place at the expense of the host cell components (11, 12).

Thus, in a manner bearing similarities to the spider and the fly, the host cell components undergo progressive disorganization and dissolution as the Bdellovibrio cell elongates to form a coiled spiral many times its initial length. This long Bdellovibrio spiral finally segments in response to some unknown signal and forms numerous small vibrioids which escape from the ghosted host cell in search of new prey. It has been observed that the flagellum and the flagellar sheath may actually form at one end of the daughter cell before it separates completely from the filamentous mother cell (3).

Within the host cell, Bdellovibrio cells find the intraperiplasmic environment nutritionally complete and the Bdellovibrio cell

need do little biosynthesis of monomeric units (17, 39, 49, 51, 52). Bdellovibrio bacteriovorus utilizes nucleoside monophosphates (52) and complete fatty acids (39) derived directly from the substrate organism for synthesis of nucleic acids and lipids. Studies have shown that the external menstrum has little influence on this life cycle. Bdellovibrio will grow in a buffer solution containing only cations and host cells (11). Thus, the host must provide all the carbon and energy sources essential for growth and reproduction of Bdellovibrio (11, 17, 39, 49, 51, 52).

The Y_{ATP} (energy efficiency = grams dry weight of cell material formed per mole of adenosine triphosphate) of intraperiplasmic growth of Bdellovibrio bacteriovorus 109J has been determined from the distribution of radioactivity of the substrate organism (U-^{14}C) E. coli, between CO_2 and Bdellovibrio cells at the end of growth (51). Unusually high values were obtained. The usual value for bacteria growing in rich medium is 10.5. A "best" Y_{ATP} value of 18.5 was obtained for Bdellovibrio parasitizing E. coli under single growth cycle conditions. An average value of 25.9 was obtained for Bdellovibrio parasitizing E. coli under conditions leading to multicyclic growth.

In their report, the authors discuss the implications of the unusually high Bdellovibrio Y_{ATP} values. A portion of this discussion follows:

> "Theoretical Y_{ATP} values are based on well established biochemical reactions (19, 53, 58). The average observed Y_{ATP} of 10.5 for many microorganisms implies that approximately 65% of the

> ATP produced by catabolism is utilized by cells in processes not accounted for by biosynthetic metabolism directly associated with the formation of cell material ... An underlying assumption in the theoretical Y_{ATP} is that catabolism and anabolism are balanced. Failure to make effective use of the ATP produced by catabolism results in energetic uncoupling. The degree of coupling between catabolic and anabolic processes may vary greatly with growth conditions (19, 53, 58). The observed Y_{ATP} may primarily reflect the overall degree of energetic coupling for a particular organism under given growth conditions."

It is suggested then:

> "... for Bdellovibrio, the degree of coupling appears quite high. This efficient coupling may result from limited energy production in the presence of excess biosynthetic metabolites. With respect to energy production, the Bdellovibrio is unique in not showing greatly enhanced respiratory rates over the endogenous rate when oxidizable substrates are available (30). In comparison with other bacteria growing on complex laboratory media, the Bdellovibrio has a much greater ability to modulate the nutritional quality of its environment. Through its regulated degradation of the substrate cell (44), the Bdellovibrio apparently controls to a large extent the types, times of appearance, rates of formation, and concentration of biosynthetic units. An accompanying regulation of energy metabolism may well explain its unusual energy efficiency."

B) Host-independent Replication

A central question concerning Bdellovibrio is the nature of its dependence upon the host cell. Host-dependent Bdellovibrio cells require host cells for growth and multiplication, even though UV-killed cells may be used. Growth and reproduction of host-dependent strains of Bdellovibrio bacteriovorus incubated with host cell-free

extracts has been demonstrated. One study (50) found that approximately five percent of a Bdellovibrio population grew into long forms in the presence of cell-free extracts of Pseudomonas, Escherichia, Micrococcus, Saccharomyces, and even Bdellovibrio. These long forms incorporated tritiated thymidine and divided under certain conditions.

A later investigation (32) demonstrated that extracts made by sonically rupturing highly concentrated suspensions of washed E. coli cells were capable of supporting growth and reproduction of a much larger portion of a Bdellovibrio inoculum. Morphological changes occurring in the cells were correlated with DNA synthesis as measured by the incorporation of radioactive precursors. This cell-free extract developmental cycle of Bdellovibrio was found to be similar to that observed in two-membered systems; early loss of flagella, elongation into filaments, and multiple fission into flagellated progeny were typical of Bdellovibrio reproduction in both systems. The ability of this extract to support Bdellovibrio reproduction was found to be stable to heat and DNase. However, treatment with Pronase or RNase destroyed the ability of the extract to support Bdellovibrio growth.

In this cell-free extract, filament length and time of division appeared to be dependent on the concentration of the host extract. In fact, dilution into extract-free medium of Bdellovibrio cells in the elongation phase of extract growth was observed to trigger multiple fission of the elongated cells. RNase treatment of the host extract

or washing the Bdellovibrio cells markedly reduced Bdellovibrio DNA synthesis and initiated Bdellovibrio division. Bdellovibrio division could be deferred and the growth cycle prolonged when additional doses of host extract were added. These observations led the authors to speculate that "growth of host-dependent Bdellovibrio is normally repressed until some product in the host or host extracts interacts with a hypothetical repressor and activates some pathway necessary for initiation of DNA synthesis." Further, by mutating from obligate to facultative host-dependency, the Bdellovibrio cells were predicated to lose repressor activity and therefore not to require an external inducer for initiation of DNA synthesis. These suggestions have not been experimentally validated.

Three methods have been used to facilitate the isolation of host-independent Bdellovibrio cells. Stolp and Starr (80) first isolated host-independent Bdellovibrios following the inoculation of concentrated suspensions (greater than 10^9 cells/ml) of host-dependent Bdellovibrio cells onto yeast-peptone agar or into nutrient broth. Shilo and Bruff (63) used a technique in which cells not attaching to host cells were concentrated and added to heavy suspensions of heat-killed host cells. The third method, developed by Seidler and Starr (57), has more consistently proven successful than the two earlier methods. In this technique, streptomycin-resistant (Sm^r) Bdellovibrio cells are isolated following infection of Sm^r host cells. These Sm^r Bdellovibrio cells are then propagated on streptomycin-sensitive (Sm^s) host cells. Finally, large numbers of these Sm^r Bdellovibrio

cells, plus some residual Sm^S host cells, are inoculated into rich medium containing streptomycin. Sm^r host-dependent Bdellovibrio cells cannot grow in the absence of host cells and Sm^r host-independent Bdellovibrio cells develop.

Bdellovibrio cells within host-independent cultures are generally quite heterogeneous with respect to cell length, motility and ability to re-establish host-dependent growth. Upon continuous cultivation away from host cells, host-independent Bdellovibrio isolates appear to lose motility and demonstrate decreased virulence for the host cell.

Although the first studies on the metabolism of Bdellovibrio were conducted using host-dependent Bdellovibrio cultures, the routine isolation of host-independent Bdellovibrio strains has greatly facilitated such studies. Metabolically Bdellovibrio cells are obligately aerobic organisms; oxygen is required for all phases of host-dependent and host-independent development (64). Bdellovibrio cells contain enzymes of the tricarboxylic acid cycle, glutamic and alanine dehydrogenases, cytochromes, nicotinamide adenine dinucleotides, and cytochrome oxidase. Bdellovibrio are almost universally negative in ability to utilize a variety of carbohydrates and some organic acids as sources of carbon and energy (57, 64). Host-independent Bdellovibrio strains demonstrate gelatine liquifaction and ammonia production (64). When first isolated they are generally catalase-positive, although this trait is often lost upon repeated transfer in host-free media (63). In general, Bdellovibrio isolates cannot

reduce nitrate although host-independent Bdellovibrio UKi2 does demonstrate nitrate reduction (15).

Seidler et al. (54) demonstrated that Bdellovibrio strains could be divided into two distinctive groups based upon the moles percent G+C contained in their DNA. Most strains were found to contain DNA of 50-51% G+C while some strains (Bdellovibrio A3.12, UKi2, and related strains) contained DNA of 42-43% G+C. Since that time further evidence has accumulated that the defining trait of Bdellovibrio "has brought together a heterogeneous assemblage of organisms" (13). Seidler et al. (13) have proposed the genus Bdellovibrio be divided into three species:

1) Bdellovibrio bacteriovorus -- with H-D Bdellovibrio bacteriovorus 100 as the nomenclatural type culture;
2) Bdellovibrio stolpii -- with H-D Bdellovibrio stolpii UKi2 as the nomenclatural type culture;
3) Bdellovibrio starrii -- with H-D Bdellovibrio starii A3.12 as the nomenclatural type culture.

Statement of goals. Members of the genus Bdellovibrio possess a life cycle unique among the bacteria. Their predatious interaction with sensitive, gram-negative bacteria initiates a complex sequence of events which, as characterized recently by Huang and Starr (35), involves "recognition by the Bdellovibrio of a congenial host, forceful physical contact of the vigorously motile Bdellovibrio cell with the other bacterial cell, attachment, penetration, digestion of the

host cell intracellular menstrum, intramural growth, and release of Bdellovibrio progeny. The mechanisms, controls, and sequence of events at play during this predatious interaction are at present only summarily delineated and are undoubtedly much more elaborate." In the belief that studies of appropriate mutant Bdellovibrio strains will aide in the further delineation and understanding of these events, in the first phase of the investigations contained within this thesis we undertook the isolation and initial characterization of temperature-sensitive mutants of host-dependent (H-D) B. bacteriovorus 109D.

The second phase of the investigations contained within this thesis was much influenced by a report of Matin and Rittenberg (44). In this publication the authors examined the kinetics of host DNA destruction and Bdellovibrio DNA synthesis through the use of a host (Pseudomonas putida N-15 - moles percent G+C = 63%), parasite (Bdellovibrio bacteriovorus 109D - moles percent G+C = 50%) system of such divergent DNA densities that the host and parasite DNAs may be easily separated in CsCl equilibrium density gradients. This novel approach prompted the suggestion that perhaps a similar technique, namely following Bdellovibrio DNA synthesis during intracellular growth via density-shift experiments, might provide a means toward new insights into Bdellovibrio physiology. Thus, it was suggested that by infecting host cells substituted with the density-label 5-bromouracil, CsCl equilibrium density gradient centrifugation could be used in a similar manner to follow the progression of host DNA destruction and Bdellovibrio DNA synthesis. This new system would

provide one important feature not present in the Pseudomonas-Bdellovibrio system. In the proposed system, newly synthesized Bdellovibrio DNA would be expected to contain the density-label in one strand only (provided Bdellovibrio DNA is replicated in a semi-conservative manner) and hence be separable from the bulk of un-replicated Bdellovibrio DNA. This feature would presumably allow one to isolate newly replicated Bdellovibrio DNA, that is to say the replication origin, and to examine this DNA in terms of its molecular biology. For example, an examination of the renaturation kinetics of replication origin DNA would presumably allow one to make certain assumptions about the control of macromolecular synthesis in Bdellovibrio, i.e., does Bdellovibrio DNA synthesis have a unique replication origin?

In this thesis, the isolation and characterization of temperature-sensitive mutants of Bdellovibrio bacteriovorus will be described. An attempt to develop a new density-label system with which to study Bdellovibrio molecular biology will be presented. And the implications of these studies on the understanding of Bdellovibrio physiology will be discussed.

MATERIALS AND METHODS

Bacterial strains. H-D Bdellovibrio bacteriovorus strain 109D and its temperature-sensitive mutant derivatives were studied. Spirillum serpens VHL was used in the preparation of all lysates and plaque-forming unit (PFU) determinations. Escherichia coli strain WP2 was used as the host strain in temperature-shift experiments and attachment kinetics experiments. Bacillus subtilis strain 168 was used in those attachment experiments noted. Escherichia coli strain UC12 (this strain also carried the designation 126) (lac^-, thy^-, str^r, $uvrA^-$, pro^-, arg^-, λ lysogen, Su^+) was used for the preparation of 5-bromouracil substituted E. coli. Bacillus subtilis 168 and E. coli UC12 were obtained from the stocks of Dr. L. R. Brown. H-I Bdellovibrio bacteriovorus 109D used in this study was originally isolated by Dr. R. J. Seidler.

Preparation of lysates and PFU enumeration. The culture medium used (designated YPS) was a modified YPSC broth (64) but prepared without cysteine and supplemented with 2 mM $MgSO_4$ and 3 mM $CaCl_2$. The stock medium (x1) contains (per liter): sodium acetate, 2 g; yeast extract (Difco), 4 g; and peptone (Difco), 4 g. The PFUs were determined, after serial dilution in x1, by using 0.8% bottom agar and 0.6% top agar of x1 YPS. Plates were incubated at 28C. For the preparation of lysates, 5 ml of an overnight S. serpens x4 YPS was added to 30 ml of x1 YPS and inoculated with 0.5 ml of Bdellovibrio. Before any Bdellovibrio culture was used for experimental purposes, a fresh lysate was prepared and transferred at least two times at

12-h intervals at 28C. Such 12-h lysates contain short, highly motile forms which are a necessity for synchronous Bdellovibrio development. Liquid cultures were shaken at 28C in a New Brunswick Scientific control environment incubator shaker at 200 rpm.

Selection of temperature-sensitive mutants. Fresh 12-h Bdellovibrio lysates were mutagenized with ethyl methane sulfonate, at a final concentration of 0.01%, for 2 to 2.5 h at 34C and then plated for isolated plaques. Replica plates were incubated at 28C and 38.5C. Lysates were made of mutants demonstrating growth at 28C but not 38.5C, and those mutants exhibiting PFU reversion frequencies of 10^{-8} or 10^{-9} were chosen for further study.

Temperature-shift experiments. A series of one-step growth experiments was run in which it was attempted to determine the time of temperature repression for each mutant (Figure 5). A fresh 12-h Bdellovibrio lysate was centrifuged at 200 x g for 10 min at 4C, sequentially filtered through a series of 5-, 3-, and 1.2-μm nitrocellulose membrane filters, and centrifuged at 25,500 x g for 15 to 20 min at 4C. Equal volumes of Bdellovibrio, resuspended in x1 to a density of 10^8 PFU/ml, and washed log-phase E. coli WP2, resuspended in x1 to a density of 5 X 10^8 cells/ml, were mixed (multiplicity of infection [MOI], 0.2) at 28C. After the infection cycle continued for some predetermined time at the permissive temperature (28C), a sample was diluted 1:10 in x1, filtered through a 1.2-μm filter, and washed with 20 ml of nutrient broth (Difco) to remove any unattached Bdellovibrio, followed by 20 ml of x1. The filter pad was inverted

over a sterile filter assembly, and cells were recovered after being washed with 10 ml of x1. A 4 ml amount of this cell suspension was added to 16 ml of x1 temperature-equilibrated at 28C or 38.5C in a bubble tube. The PFUs per milliliter were then followed hourly for 6 h, and the burst size and percentage of increase were determined as described.

Attachment kinetics determinations. Attachment kinetics were studied by using a modification of the procedure described by Varon and Shilo (87). In our experiments, 10^8 Bdellovibrio and 5 x 10^8 E. coli as described above, or 2 x 10^8 washed and x1-resuspended log-phase B. subtilis, were mixed after preincubation for 10 min at the appropriate temperature. Immediately after mixing and at time intervals thereafter, a sample was withdrawn and diluted 1:100 in chilled x1. After the last sample was withdrawn, 1 ml of each dilution was filtered through a 1.2-μm filter and washed with 9 ml of x1, and the filtrate PFUs were determined.

Isolation and growth of host-independent strains of 109D3 and 109D48. Host-independent strains of the temperature-sensitive Bdellovibrio bacteriovorus 109D mutant derivatives 109D3 and 109D48 described herein were isolated following their inoculation upon lawns of heat-killed Spirillum serpens VHL. Spirillum serpens x4 YPS cultures were harvested by centrifugation and resuspended in peptone-yeast extract medium (PYE, peptone 10 g/l; yeast extract 3 g/l) to an A_{600} of 10-20. This heavy cell suspension was then placed in a live steam chamber for 20 min. No survivors were noted after 48-h

incubation at 28C when 0.2 ml was spread onto the surface of a PYE agar (1.5% agar) plate. For the isolation of host-independent strains, one ml of this heat-killed S. serpens preparation was added to a 1.5 ml PYE agar overlay (2% agar) tube and 0.5 ml of an appropriate dilution (x1) of a Bdellovibrio concentrate (high speed pellet after sequential filtration of a fresh lysate) was added. The tube was rapidly mixed and poured onto the surface of a PYE agar plate. After prolonged incubation at 28C (6-15 days), small yellow colonies of H-I Bdellovibrio cells appeared. These were inoculated into, and maintained thereafter in, PYE.

Measurement of macromolecular capabilities. The ability of a particular S. serpens-Bdellovibrio combination to synthesize ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) was followed by measuring the incorporation of [^{3}H]uracil or [^{3}H]thymidine into cold trichloroacetic acid-precipitable counts. For these assays, a 0.1 ml sample of the cell suspension was mixed at the time indicated with 1 ml of 5% cold trichloroacetic acid and allowed to stand at 4C for 30 min. The precipitates formed were collected and washed on a 1.2-μm nitrocellulose membrane filter with 10 ml of cold 5% trichloroacetic acid, followed by 10 ml of cold 70% ethanol. In control experiments, S. serpens was found not to incorporate extracellular [^{3}H]thymidine. Therefore, for the assay of DNA synthesis, 1.2×10^9 S. serpens cells that were washed and suspended in a Tris(hydroxymethyl)aminomethane (0.001 M at pH 7.5)-calcium/magnesium salts (2 and 3 mM, respectively) buffer (TCM buffer) were mixed with three

times as many filtered and washed Bdellovibrio cells in TCM buffer containing either 0.2 or 0.4 μCi of [^{3}H]thymidine per ml. For the assay of RNA synthesis, 1.2 X 10^9 washed and resuspended S. serpens cells were preincubated for 30 min in TCM buffer containing 0.2 or 0.4 μCi of [^{3}H]uracil per ml prior to the addition of the Bdellovibrio cells. Such [^{3}H]uracil-preincubated S. serpens cells did incorporate some cold trichloroacetic acid-precipitable counts, but in control experiments this incorporation was shown to cease after 15 min of incubation in the absence of Bdellovibrio. The release of extracellular, Bdellovibrio-specific proteases was followed via Azocollase activity by the method of Gloor et al. (20). Increase in extracellular proteolytic activity is attendant with intracellular Bdellovibrio growth and represents the release of protease unit values far and above those found in host cell cultures or host cell extracts and, as such, is taken to represent the induction of a Bdellovibrio-specific enzyme system.

Electron microscopy. Two-membered cultures of wild-type 109D, 109D3, and 109D48 were prepared (at an MOI of 3) with E. coli WP2 as host. After being shaken at the nonpermissive temperature for 60 min, each culture was fixed by the modified Ryter and Kellenberger procedure of Brock and Edwards (6). Cell pellets were dehydrated through a graded series of water-ethanol mixtures followed by propylene oxide. They were infiltrated with a propylene oxide-Epon-Araldite mixture and embedded in Epon-Araldite by conventional methods. Sections were double-stained with uranyl acetate and lead

citrate and examined in a Phillips EM 200 electron microscope at 60 kV.

Preparation of 5-bromouracil substituted E. coli. 5-Bromouracil (5BU) substituted E. coli were prepared by a modification of the technique of Hanawalt (28). E. coli UC12 were shaken overnight in PYE at 37C and then 0.5 ml transferred to 100 ml of thymine supplemented minimal medium. This minimal medium consisted of the following components:

A. Tris buffer and salts (adjusted to pH 7.4 in 1 liter of distilled water by addition of HCl): Tris (hydroxymethyl) aminomethane, 12 g; KCl, 2 g; NH_4Cl, 2 g; $MgCl_2 \cdot 6\ H_2O$, 0.5 g; Na_2HPO_4, 0.35 g; Na_2SO_4, 0.35 g;

B. Supplements (stock solutions prepared at 100 X concentration and filter sterilized): uracil, 10 µg/ml; arginine, proline, methionine, and tryptophan, 20 µg/ml; thiamine and biotin, 2 µg/ml;

C. Energy source: 0.5% glucose in medium (stock solution, 25% glucose in distilled water);

D. Thymine, 2 µg/ml in medium (stock solution prepared at 100 X concentration and filter sterilized);

E. 5-Bromouracil, 10 µg/ml in medium (weighed and added to unsupplemented medium at least 24 h prior to use).

The components A, B, and C are prepared separately, A and C being sterilized by autoclaving and then added as required. Component D or component E is then added as noted for the complete culture

medium.

Following inoculation into thymine supplemented minimal medium, growth at 37C was closely monitored via optical density readings at 600 nm (A_{600}) and the A_{600} maintained below 0.4 by periodic transfer into fresh, prewarmed thymine supplemented minimal medium. After at least three transfers in thymine supplemented minimal medium, cells were harvested via centrifugation at 9500 x g for 10 min at 5C and resuspended in prewarmed 5BU supplemented minimal medium to an A_{600} of approximately 0.1 and shaken vigorously at 37C for 120 min. Cells were then harvested via centrifugation as before and held at 4C until used.

Preparation of isotopically labeled Bdellovibrio bacteriovorus 109D. Isotopically labeled Bdellovibrio cells were obtained by initially supplementing a 12-h Spirillum serpens lysate preparation with 1 µCi/ml 6-^{3}H-thymidine (25 Ci/mM). Following lysis, a 200 x g, 10-15 min, refrigerated low speed centrifugation was performed to remove unlysed host cells. The supernatant solution was then filtered sequentially through a 5, 3 and 1.2 µm porosity millipore membrane filter series and this filtrate subjected to a 25,000 x g, 20 min, refrigerated centrifugation. The Bdellovibrio pellet was resuspended in a 1 to 10 dilution of PYE supplemented with 2 mM $MgSO_4$ and 3 mM $CaCl_2$ (PYE/10) to an A_{600} of 1.5 and used as described.

Preparation of cell lysates for analysis by CsCl equilibrium density gradient centrifugation. Isotope incorporation and growth were stopped abruptly by the dilution of a culture with an equal

volume of ice-cold buffer (containing 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris(hydroxymethyl)aminomethane pH 8.0 and 0.01 M KCN) termed killing solution. Cells were harvested by refrigerated centrifugation at 12,000 x g for 15 min and frozen at -20C. Cell pellets were subsequently thawed and resuspended in a minimal volume of saline-EDTA (0.15 M NaCl and 0.015 M EDTA pH 8.0). DNA preparations designated as highly purified were essentially prepared as per Marmur (43). In this procedure cell lysis was achieved by the addition of sodium lauryl sulfate (SLS) to a final concentration of 2%. This cell lysate was treated with 200 µg/ml RNase (pretreated by heating at 80C for 10 min) for 1 h at 37C followed by treatment with 100 µg/ml self-digested pronase for 1 h at 37C. Following extraction with saline-sodium citrate (0.15 M NaCl, 0.015 M Na citrate at pH 8.0 [SSC]) equilibrated phenol, the aqueous phase was deproteinized via 2-3 extractions with chloroform-isoamyl alcohol (24/1) (59). Two volumes of chilled ethanol were gently layered onto the solution and DNA was collected by spooling onto a glass rod slowly rotated at the aqueous-ethanol interface. Spooled DNA was redissolved in 0.1 X SSC, adjusted to 1 X SSC and resubmitted to spooling via ethanol precipitation as before. Following resuspension in 0.1 X SSC, a final spooling was achieved by the addition of Na acetate to 0.3 M and the precipitation of DNA by dropwise addition of 0.6 volumes of isopropanol. This highly purified DNA was resuspended in 0.1 X SSC and stored at 4C over $CHCl_3$.

DNA samples not designated as highly purified were lysed by the

addition of sodium lauryl sarcosinate (NP-97) to a final concentration of 2%. Following sequential treatment with RNase (200 µg/ml) and Pronase (100 µg/ml) for 1 h at 37C and deproteinization with a one-to-one mixture of SSC equilibrated phenol-sevag, samples were dialyzed against two changes of 1 X SSC. For CsCl equilibrium density centrifugation, 5.55 g of solid CsCl (Kawecki Berylco Industries, Inc., technical grade) was weighed into tared ultracentrifuge tubes and 4 ml of sample added. This technique yields 5.2 ml of solution with an initial mean density of approximately 1.73 g/ml. Centrifugation was carried out using noryl capped thick-walled polycarbonate ultracentrifuge tube assemblies (Beckman #336479) in a Type 50 rotor at 40,000 rpm for greater than 40 h at 26C in a Beckman Spinco L2-65 or L2-50 ultracentrifuge.

Following centrifugation, fractions were collected from the top of the gradient using a Buchler Auto Densi-Flow #C gradient collector (Buchler Instruments, Div. of Searle Analytic, Inc.). Fractions were collected directly onto prepunched and numbered cellulose filter paper discs (Whatman No. 1, one inch in diameter) and assayed for trichloroacetic acid (TCA) precipitable counts by the method of Bollum (5). In this procedure, discs with spotted and dried fractions were collected into chilled 5% TCA containing 1% $Na_4P_2O_7 \cdot 10H_2O$ and allowed to stand with gentle mixing of 15 min. Following a second 15 min, chilled 5% TCA wash, fractions were washed twice with chilled 95% ethanol (15 min/wash) and air dried. Dried filters were counted via liquid scintillation while lying under 5 ml of toluene-Omnifluor (Beckman).

Prevention of photobromolysis. It should be noted that all cultures containing 5BU were kept in the dark and handled only with the room lights off. DNA purifications were carried out in a room equipped with fluorescent lamp bulbs having yellow glass envelopes.

RESULTS

Bacterial enumeration. In the following studies, host bacterial strains (Spirillum serpens VHL and Escherichia coli WP2) were grown in x4 YPS culture medium at 28C as described (Materials and Methods). to aid in the enumeration of host cells in further experiments, standard curves correlating the absorbance at 600 nm (A_{600}) and viable cell counts of washed, resuspended host cell preparations were determined. The relationships between A_{600} and viable cell numbers obtained for S. serpens and E. coli are illustrated in Figure 2 and Figure 3, respectively.

A standard curve relating A_{600} and plaque-forming units (PFUs) of B. bacteriovorus 109D was also prepared. Before any Bdellovibrio culture was used for experimental purposes, a fresh lysate was prepared and transferred at least two times at 12-h intervals at 28C. Such 12-h lysates contain short, highly motile forms which are a necessity for synchronous Bdellovibrio development. It is doubly important that Bdellovibrio preparations be harvested under standardized conditions, since further incubation leads to an increase in cell length (A_{600}) without a concomitant increase in cell numbers (PFUs). The relationship between A_{600} and PFUs obtained for B. bacteriovorus 109D is illustrated in Figure 4.

Figure 2. Absorbance at 600 nm *vs.* viable cell numbers of *Spirillum serpens* VHL.

A 12-h x4 YPS *S. serpens* VHL culture grown as described (Materials and Methods) was centrifuged at 12,000 x g for 10 min and the cell pellet resuspended in x1 YPS. The A_{600} of this suspension and various dilutions of it were determined using a Coleman Spectrophotometer 20. Viable cell counts of each cell suspension were determined following serial dilution in x1 YPS and plating on x4 YPS.

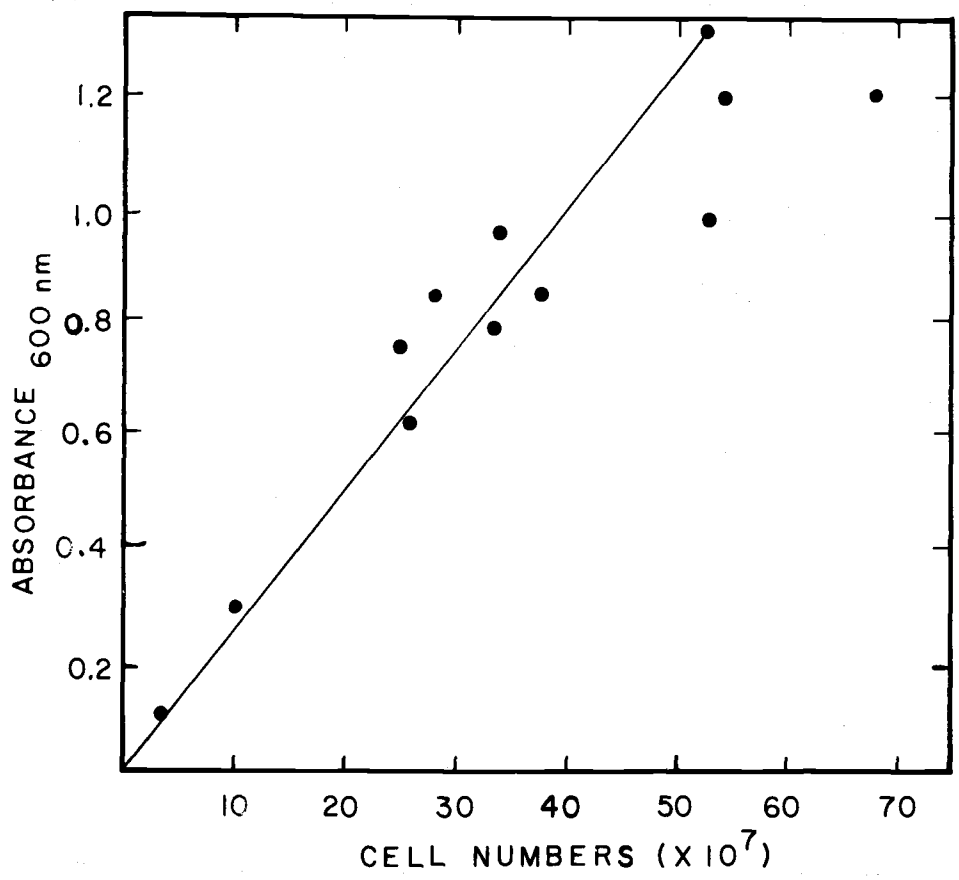

ABSORBANCE 600 nm
1.2
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
60
70
CELL NUMBERS (X 10^7)

Figure 3. Absorbance at 600 nm *vs.* viable cell numbers of *Escherichia coli* WP2.

A 12-h x4 YPS *E. coli* WP2 culture was grown as described (Materials and Methods) and centrifuged at 12,000 x g for 10 min and the cell pellet resuspended in x1 YPS. The A_{600} of this suspension and various dilutions of it were determined using a Coleman Spectrophotometer 20. Viable cell counts of each cell suspension were determined following serial dilution in x1 YPS and plating on x4 YPS.

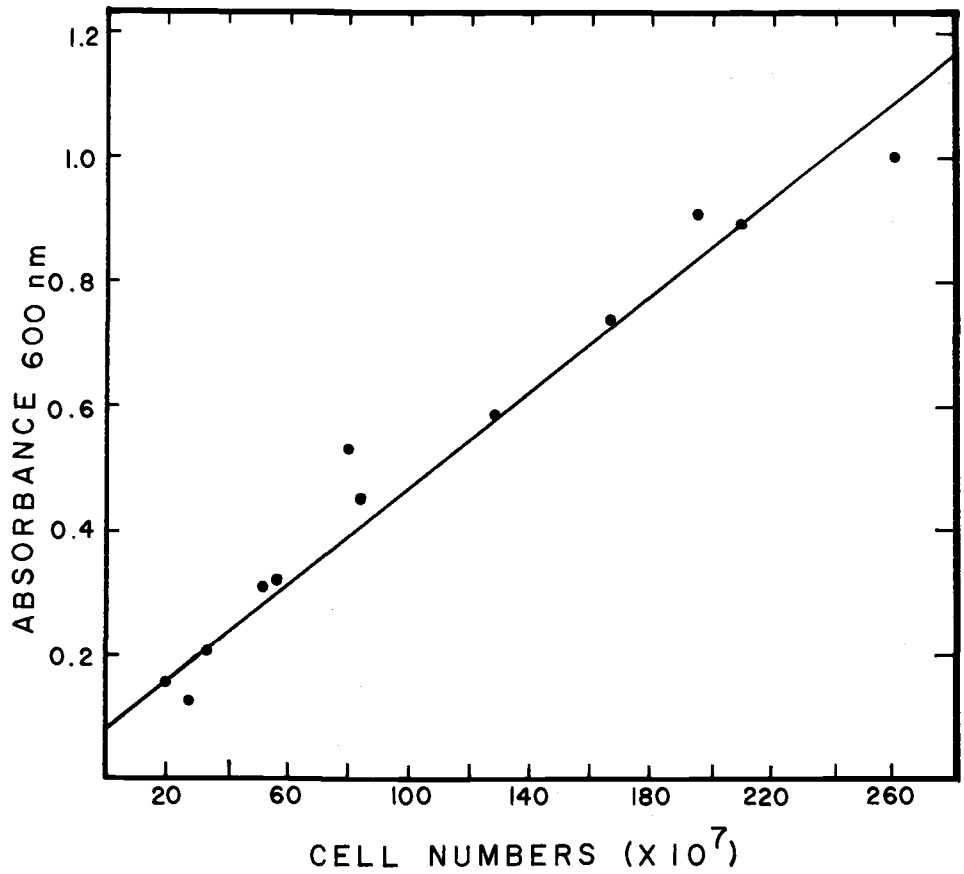
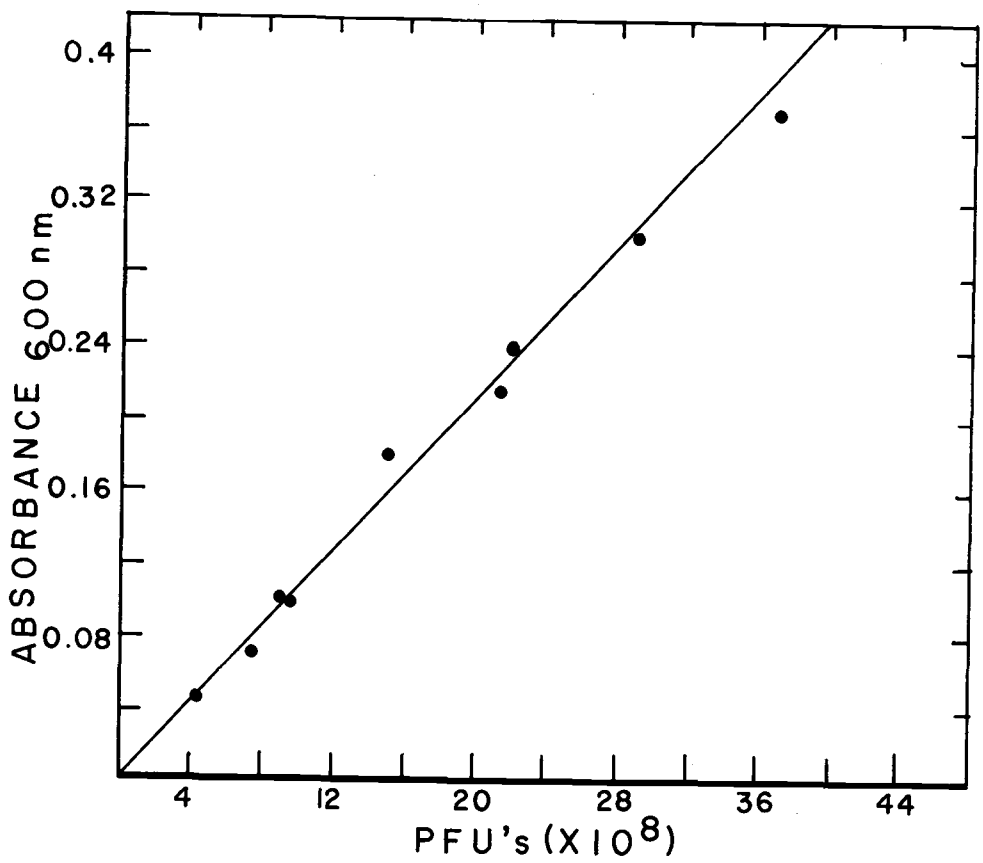

ABSORBANCE 600 nm
1.2
1.0
0.8
0.6
0.4
0.2
20
60
100
140
180
220
260
CELL NUMBERS (X 10^7)

Figure 4. Absorbance at 600 nm vs. plaque forming units of Bdellovibrio bacteriovorus 109D.

A 12-h S. serpens VHL - Bdellovibrio bacteriovorus 109D lysate was prepared as described (Materials and Methods). Following a low speed centrifugation (200 x g for 10 min), the supernatant solution was sequentially passed through a graded 5, 3, 1.2 μm millipore membrane filter series. The final filtrate was centrifuged (25,500 x g for 20 min) and the cell pellet resuspended in x1 YPS. The A_{600} and PFUs of this suspension and dilutions of it were determined as described (Materials and Methods).


0.4
0.32
0.24
0.16
0.08
ABSORBANCE 600 nm
4
12
20
28
36
44
PFU's (X 10^8)

For mutagenesis, fresh 12-h Bdellovibrio lysates were treated with EMS at a final concentration of 0.01%, for 2 to 2.5 h at 34C, and then plated for isolated plaques. Replica plates were produced by transfering inocula from isolated plaques to each of two plates containing lawns of S. serpens. Replica plates were incubated at 28C and 38.5C. Replica plates were examined following 3-6 days' incubation. Lysates were made of mutants demonstrating growth at 28C but not 38.5C. Serial dilutions were made and plated in duplicate and replicate sets incubated at 28C and 38.5C. Only those mutants exhibiting PFU reversion frequences of 10^{-8} or 10^{-9} were chosen for further study.

Preliminary characterization. Preliminary characterizations of those mutants selected for further study were made by microscopic obervation of synchronously infected host cells incubated at 38.5C. Mutants were examined for motility, the ability to carry out attachment, the formation of host cell spheroplasts and the progression of intramural development at the nonpermissive temperature. Following microscopic observation at the nonpermissive temperature, mutants could generally be defined as exhibiting either early or late blockage. Some mutants could be tentatively assigned as possessing a particular stage of blockage. Thus, a mutant designated 109D153 was motile at the nonpermissive temperature but failed to produce host cell

spheroplasts. Some apparent attachments were observed but at a frequency less than normal. This mutant was then tentatively classified as attachment deficient. Mutants designated 109D3 and 109D48 were motile at the nonpermissive temperature and appeared to attach normally to host cells. However, they failed to produce host cell spheroplasts at 38.5C. Mutants designated 109D4 and 109D152 were motile and appeared to attach normally at the nonpermissive temperature. Host cell spheroplasts were produced though no increase in PFUs was observed upon continued incubation at 38.5C.

The mutants described above (109D153, 109D3, 109D48, 109D4 and 109D152) were selected as demonstrating reversion frequencies of 10^{-8} or 10^{-9} with the lawn overlay technique described. They also all failed to yield PFU increases when 38.5C, two-membered cultures were prepared by mixing temperature-equilibrated host and Bdellovibrio cells. Mutants, however, were isolated that met the first criterion but not the second. This class of organisms apparently represents mutants which can initiate at least partial rounds of replication before the effects of the deficiency expressed at nonpermissive temperatures are sufficiently felt to prevent further replication.

Time of temperature repression. In two-membered x1 YPS, 28C cultures containing E. coli WP2 and B. bacteriovorus 109D (present at a multiplicity of infection [MOI] of 0.2), attachment is virtually complete by 8 min. By phase-contrast microscopy, host cell spheroplasts appear within 15 min and penetration of Bdellovibrio between the host cell wall layers and membrane is completed by about 1 h.

Bdellovibrio PFUs begin to increase after 3 to 4 h incubation. Under these conditions, then, the Bdellovibrio predatory growth cycle may be chronologically subdivided into three sequential steps: i) attachment, which is normally completed rapidly; ii) penetration, which is complete by approximately 1 h after infection; and iii) intracellular development, which occurs from approximately 1-4 h post-infection.

A series of temperature-shifted, one-step growth experiments was run with each mutant to attempt to delineate the time of release from temperature repression for that mutant. The protocol used for these experiments is illustrated in Figure 5. E. coli WP2 and the Bdellovibrio mutant under investigation were mixed at an initial MOI of 0.2 at the permissive temperature. At some predetermined time into the infection cycle, portions of this culture were removed and transferred to the permissive and nonpermissive temperatures under conditions which allow for one-step growth (56). Failure of the portion shifted to the nonpermissive temperature to yield a significant burst (less than 100% PFU increase) was taken as evidence that the time of the temperature shift was prior to the completion of the temperature-sensitive event. The onset of an increased burst was taken to denote the time period required for release from temperature sensitivity.

As may be seen in Figure 6, the percent increases exhibited at 28C vary from mutant to mutant, though mutants rarely yielded percent increases less than a cell doubling (100% in Figure 6). Mutant 109D4 demonstrated the smallest permissive temperature percent increase observed -- about 200%. Mutants 109D3, 109D48, and 109D152 demon-

Figure 5. Experimental procedure for temperature-shifted one-step growth experiments.

The procedure outlined was designed to allow shift-up experiments to be run under conditions permitting the one-step growth of Bdellovibrio bacteriovorus 109D. In this procedure, Bdellovibrio and E. coli cells were mixed at a low multiplicity of infection. After a predetermined time at 28C, unattached Bdellovibrio cells were removed by the filtration technique of Varon. Infected and uninfected host cells were then recovered from the filtration membrane. Half of the recovered cells were incubated further at 28C, while the second half were incubated at 38.5C.

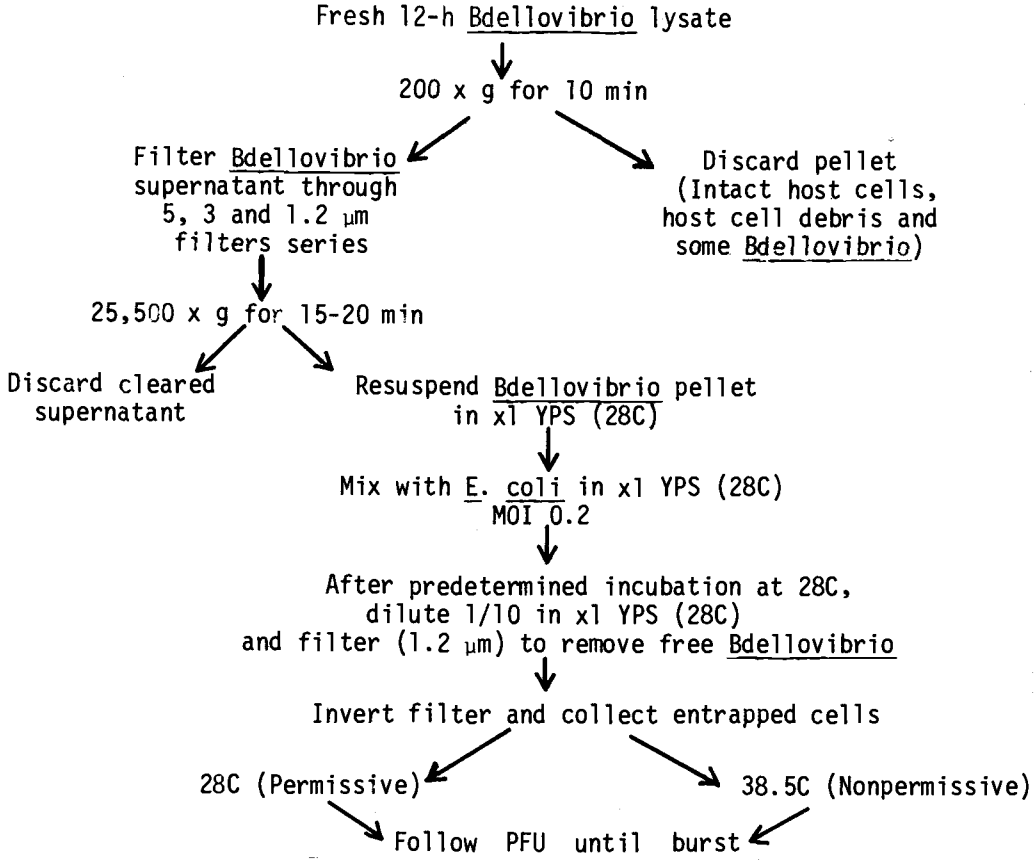
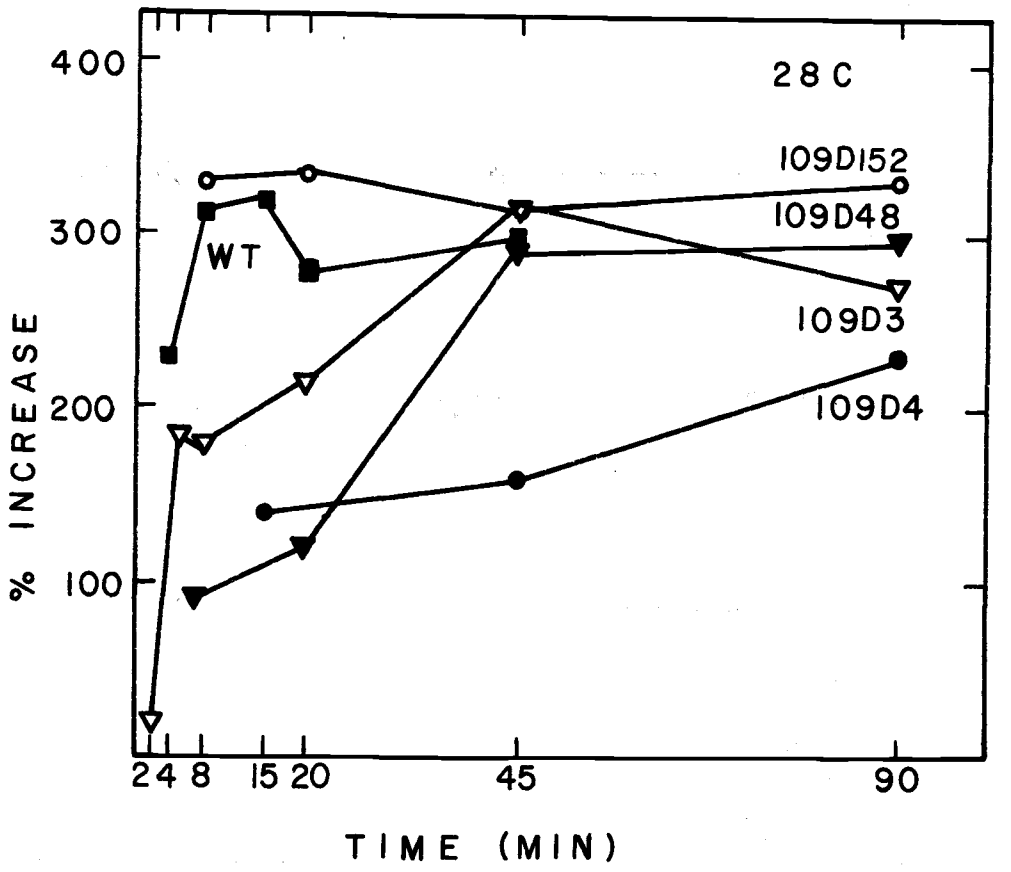

Fresh 12-h Bdellovibrio lysate
200 x g for 10 min
Filter Bdellovibrio supernatant through 5, 3 and 1.2 µm filters series
Discard pellet (Intact host cells, host cell debris and some Bdellovibrio)
25,500 x g for 15-20 min
Discard cleared supernatant
Resuspend Bdellovibrio pellet in x1 YPS (28C)
Mix with E. coli in x1 YPS (28C) MOI 0.2
After predetermined incubation at 28C, dilute 1/10 in x1 YPS (28C) and filter (1.2 µm) to remove free Bdellovibrio
Invert filter and collect entrapped cells
28C (Permissive)
38.5C (Nonpermissive)
Follow PFU until burst

Figure 6. One-step growth of wild-type or mutant Bdellovibrio bacteriovorus 109D maintained at 28C.

Percent PFU increases observed when two-membered cultures of wild-type or mutant B. bacteriovorus 109D and E. coli WP2 are grown under conditions which allow for one-step growth. The protocol followed is illustrated in Figure 5. The times indicated represent the interval of incubation at 28C prior to the elimination of unattached Bdellovibrio cells via filtration. Symbols: ■ , wild-type 109D; ▼ , 109D48; ● , 109D4; ○ , 109D52.


400
300
200
100
% INCREASE
28 C
109D152
109D48
WT
109D3
109D4
2 4 8 15 20 45 90
TIME (MIN)

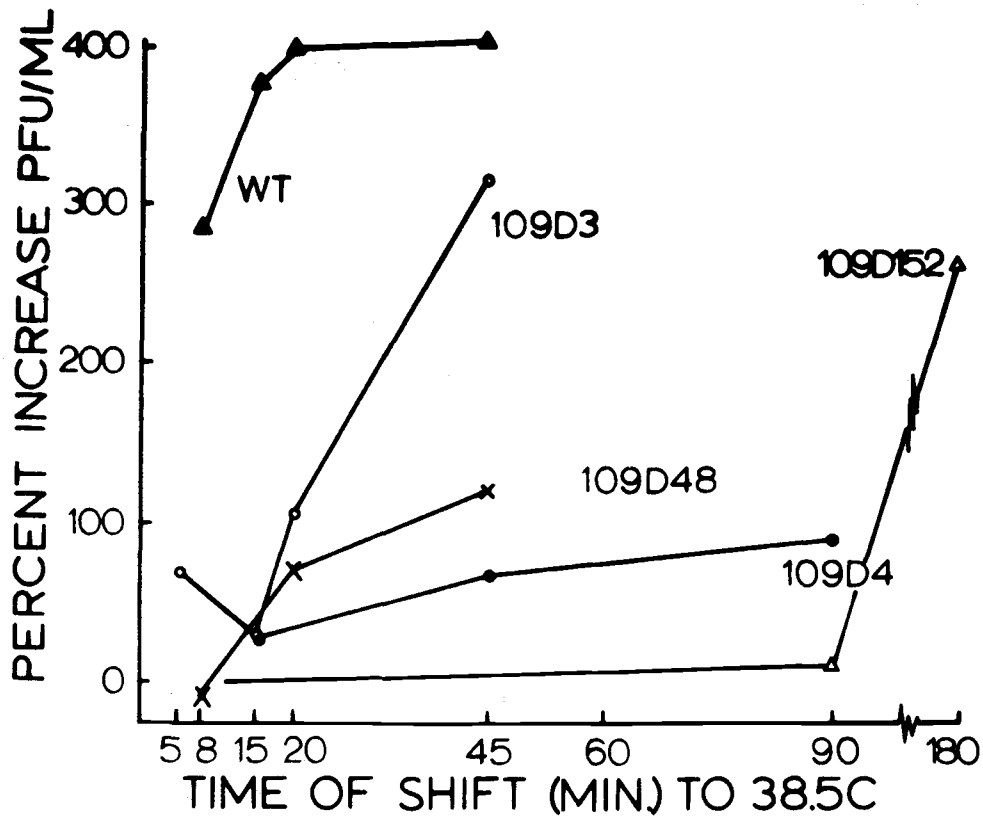
strated percent increase values of 270, 290, and 330%, respectively.

It is evident in Figure 6 that the time chosen to carry out the manipulations involved in washing free unattached Bdellovibrio cells affects the final percent increase achieved, even though the cells are continually maintained at the permissive temperature (28C). The percent increase value calculation is based upon the assumption that all the infecting Bdellovibrio cells attach. The low percent increase values seen at early time periods (0-10 min) in both the wild-type and mutant infections may be partially due to the failure of the entire inoculum to attach. However, some mutants, 109D48 for example, demonstrate repressed growth (decreased percent increases upon burst) even when incubated for as long as 20 min before the unattached Bdellovibrio cells are washed. As will be illustrated later, these mutants invariably demonstrate normal attachment kinetics at the permissive temperature (28C) and failure of a significant proportion of the population to attach would not seem to be a plausible explanation for the repressed growth illustrated. This permissive temperature growth repression was not clarified further, though it obviously is characteristic of the particular mutation involved since wild-type burst sizes rapidly stabilize and different mutants exhibit differing patterns of growth repression.

The growth increments observed at the nonpermissive temperature for the mutants were examined (Figure 7). Release from temperature repression to yield PFU increases greater than a doubling is illustrated by 109D3 at 20 min, but not at 15 min, into its

Figure 7. One-step growth of wild-type or mutant Bdellovibrio bacteriovorus 109D following shift-up.

Percent PFU increases observed when a two-membered culture of wild-type or mutant B. bacteriovorus 109D and E. coli WP2 is shifted at the times indicated from the permissive temperature (28C) to the nonpermissive temperature (38.5C) under conditions which allow for one-step growth. The protocol followed is illustrated in Figure 5. Symbols: ▲, wild-type 109D; O, 109D3 (penetration); X, 109D48 (penetration); ●, 109D4 (intracellular); △, 109D152 (intracellular).

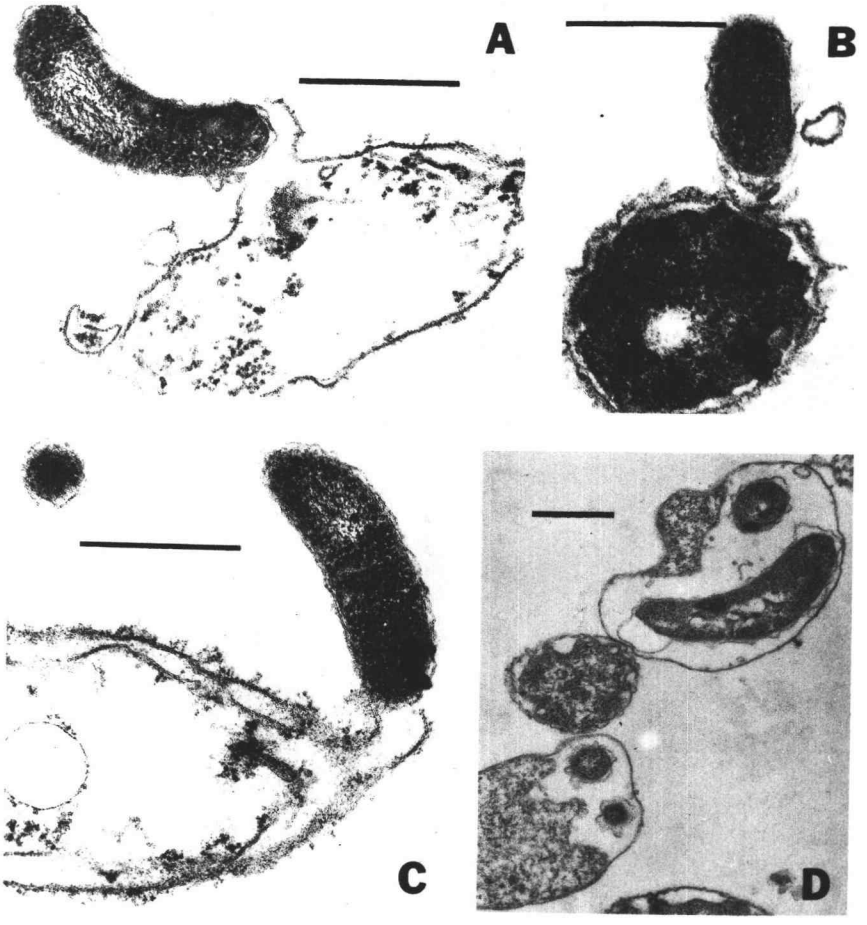

PERCENT INCREASE PFU/ML
400
300
200
100
0
WT
109D3
109D152
109D48
109D4
5 8 15 20
45
60
90
180
TIME OF SHIFT (MIN.) TO 38.5C

infection cycle. Mutant 109D48 is capable of greater than a cell doubling when shifted to 38.5C at 45 min into its infection cycle. When shifted at 15 min, only a 70% increase occurs, whereas at 8 min a slight drop in PFUs is recorded. Both 109D3 and 109D48 were identified by phase-contrast microscopy as mutants capable of attaching to, but not able to penetrate, the host cell wall. Mutant 109D152 is not able to multiply even when shifted to 38.5C at 90 min into its infection cycle, but when shifted at 180 min it is capable of a significant burst (Figure 7). When shifted to the nonpermissive temperature as late as 90 min into its infection cycle, mutant 109D4 is capable of only an 85% increase in Bdellovibrio PFUs. Both 109D4 and 109D152 were identified by phase-contrast microscopy as mutants blocked at some intracellular developmental stage.

Electron microscopy of penetration-deficient mutants. Nonpermissive cultures of 109D3 and 109D48, the two penetration-deficient mutants described, were examined by electron microscopy. Temperature-equilibrated mutant and E. coli WP2 cultures were mixed at an MOI of 3, shaken at 38.5C for 60 min, and then fixed as described (Materials and Methods). Figure 8 (A, B, C) illustrates the failure of 109D3 and 109D48 to initiate host cell penetration after 60 min at the nonpermissive temperature. Wild-type 109D cells have completely invaded the host (Figure 8D). It is of interest to note the extent of host cell dissolution which is evident in both mutant preparations. It is impossible to tell from the present study whether this dissolution is due to Bdellovibrio lytic systems or host

Figure 8. Electron microscopy of Bdellovibrio bacteriovorus 109D penetration defective mutants.

Bdellovibrio-E. coli two-membered mixtures (38.5C) incubated for 60 min and then fixed for electron microscopy. Bar represents 0.5 μm in all micrographs. (A, B) 109D3: Note extracellular position of Bdellovibrio. Host cell wall rupture exhibited in A is probably in part due to the advanced degree of host degradation demonstrated. The majority of host cells appeared to be undergoing dissolution (A, C), although some host cells still retain a more organized state (B). Magnification, x50,500. (C) 109D48: Note failure of Bdellovibrio to penetrate. Magnification, x50,500. (D) Wild-type 109D: Note fully penetrated, intracellular position of Bdellovibrio cells. Magnification, x26,000.


A
B
C
D

cell autolytic enzymes. It should be noted, however, that although 109D3 and 109D48 fail to exhibit PFU increases at the nonpermissive temperature, they obviously are capable of causing host cell death.

HI-109D48. Several investigators have suggested that Bdellovibrio penetration of the host bacterium may be aided by the action of one of the several Bdellovibrio proteases. If this is the case, mutant 109D48 might produce detectably decreased protease levels at the nonpermissive temperature. To investigate this possibility, host-independent (HI) strains of wild-type 109D and 109D48 were examined for Azocollase activity at 38.5C. Azocollase assays were kindly performed by Brian Klubeck. No difference in the levels of total Azocollase activity was detected. This result was not surprising since the HI-109D48 isolate was found to continue growth at the nonpermissive temperature. Figure 9 illustrates the growth of HI-109D48 at 38.5C. The A_{600} of HI-109D48 is seen to continue to increase at 38.5C, although at a diminished rate when compared with the increase in A_{600} of HI-109D48 at the permissive temperature or the increase in A_{600} demonstrated by the wild-type host-independent isolate at 28C and 38.5C.

Attachment kinetics. The ability to follow Bdellovibrio attachment to E. coli via differential filtration through a membrane filter has been demonstrated (84) and was used in this study to follow the attachment kinetics of 109D wild-type and mutant Bdellovibrio. Figure 10 illustrates the attachment kinetics of wild-type B. bacteriovorus strain 109D to E. coli WP2 at 38.5C. The kinetics observed

Figure 9. Growth of H-I Bdellovibrio bacteriovorus 109D48 at 38.5C.

A host-independent strain of Bdellovibrio bacteriovorus mutant 109D48 was isolated (Materials and Methods) and its growth response at 28C and 38.5C examined. Turbid cultures of H-I B. bacteriovorus 109D and H-I B. bacteriovorus 109D48 were diluted 1/10 into fresh medium and their A_{600} determined periodically upon continued incubation at 28C. At the time indicated by the arrow, one-half of each culture was shifted to 38.5C.

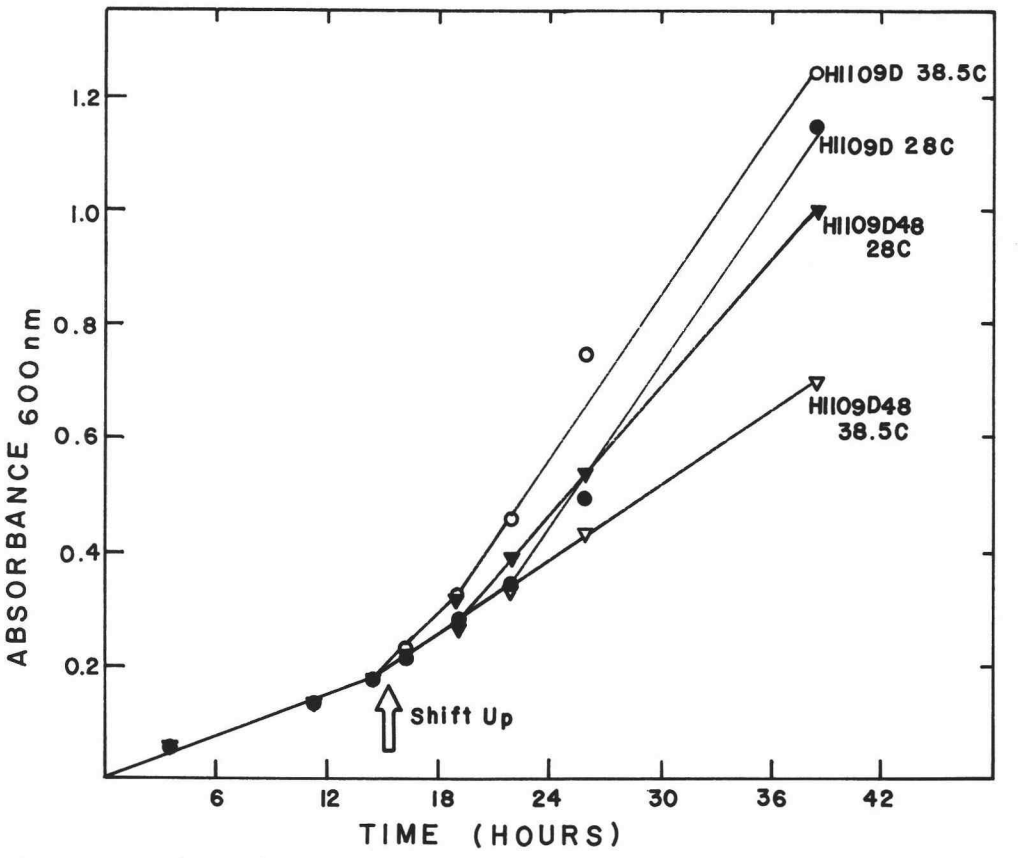
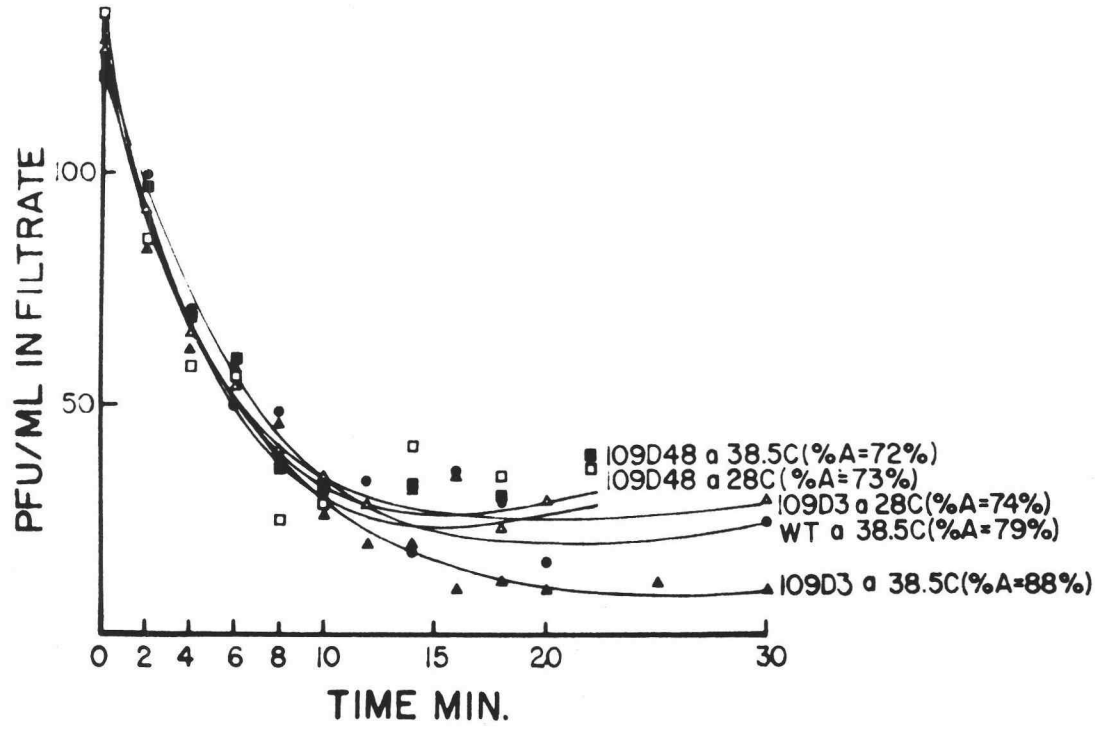

HI109D 38.5C
HI109D 28C
HI109D48 28C
HI109D48 38.5C
Shift Up
ABSORBANCE 600 nm
TIME (HOURS)
0.2
0.4
0.6
0.8
1.0
1.2
6
12
18
24
30
36
42

Figure 10. Attachment kinetics of 109D3 and 109D48 to E. coli WP2.

The attachment kinetics of 109D3 and 109D48 to E. coli WP2 at the permissive (28C; △ , □) or the nonpermissive (38.5C; ▲ , ■) temperatures are illustrated. The attachment kinetics of wild-type 109D to E. coli WP2 at 38.5C is also presented (●). The rate of decrease in the numbers of Bdellovibrio cells passing a 1.2 μm filter is presented.

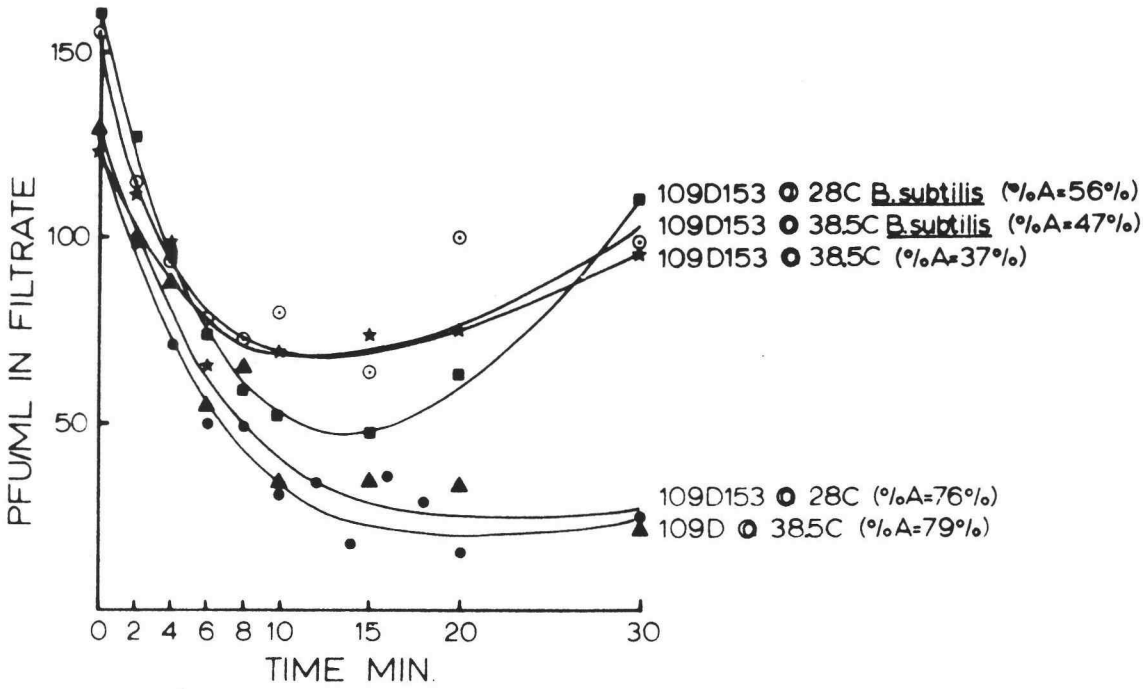

PFU/ML IN FILTRATE
100
50
0 2 4 6 8 10 15 20 30
TIME MIN.
109D48 a 38.5C(%A=72%)
109D48 a 28C (%A=73%)
109D3 a 28C(%A=74%)
WT a 38.5C(%A=79%)
109D3 a 38.5C(%A=88%)

compare favorably with those recorded by Varon and Shilo (87). By 10 min, the drop in PFUs obtained in the filtrate has essentially stabilized. If the average of the points taken after the 10 min sample is used in the calculation, attachment is shown by 79% of the Bdellovibrio. Penetration mutant 109D3 (Figure 10) demonstrates attachment similar to that of the wild-type, yielding values of 74 and 88%, respectively, at the permissive and nonpermissive temperatures. Another penetration mutant, 109D48, demonstrated similar kinetics, with the final attachment calculations yielding values of 73 and 72% (Figure 10). Two mutants which possess stages of blockage relatively late in the infection cycle, 109D4 and 109D152, demonstrate unimpaired attachment kinetics at the nonpermissive temperature. The curves are not illustrated since the attachment kinetics observed were virtually identical to those of Figure 10. The attachment calculations yield values of 77 and 65%, respectively.

Some degree of difficulty was experienced in attempting to isolate mutants which would fit our preconceived idea of an attachment-negative mutant. When assessed by phase-contrast microscopy at the nonpermissive temperature, all mutants demonstrated visible attachment. With 109D153 it was noted, however, that attachment was less efficient than normal, and further investigations of this strain were initiated. The attachment kinetics of 109D153 to E. coli and B. subtilis at the permissive and nonpermissive temperatures is shown in Figure 11. Attachment to E. coli at the permissive temperature follows wild-type kinetics and yields a final attachment calculation

Figure 11. Attachment kinetics of 109D153 to E. coli WP2 and B. subtilis 168.

The attachment kinetics of 109D153 to E. coli WP2 at the permissive (28C; ▲) and the nonpermissive (38.5C; ★) temperatures and the attachment kinetics of 109D153 to Bacillus subtilis at the permissive (28C; ■) and the nonpermissive (38.5C; O) temperatures are presented. The wild-type attachment kinetics at 38.5C is also presented (●).


150
100
50
PFU/ML IN FILTRATE
0 2 4 6 8 10 15 20 30
TIME MIN.
109D153 ⊙ 28C B.subtilis (%A=56%)
109D153 ⊙ 38.5C B.subtilis (%A=47%)
109D153 ⊙ 38.5C (%A=37%)
109D153 ⊙ 28C (%A=76%)
109D ⊙ 38.5C (%A=79%)

value of 76%. The attachment kinetics of 109D153 to E. coli at the nonpermissive temperature are significantly depressed, and incubation of the chilled attachment mixture appears to facilitate detachment. When the final attachment calculations as described above are applied to 109D53-E. coli mixtures at the nonpermissive temperature, a value of 37% is obtained.

Microscopic observations have shown that Bdellovibrio exhibits attachment to infected spheroplasts, gram-positive bacteria, cover slips, etc. These observations lead to the conclusion that Bdellovibrio is capable of a degree of nonspecific attachment. The application of this conclusion to 109D153 led us to examine the attachment kinetics of 109D153 to B. subtilis at the permissive and nonpermissive temperatures. These attachment kinetics illustrated in Figure 11 are strikingly similar to the attachment kinetics of 109D153 to E. coli at the nonpermissive temperature. The attachment values are 56 and 47% for the permissive and nonpermissive temperatures, respectively. Note also that incubation of the attachment mixture in ice appears to reveal detachments of the nonspecific type; thus, filtrate PFUs rise in the latter portions of the B. subtilis-109D153 and E. coli-109D153 nonpermissive attachment curves but remain flat in the other attachment curves. These observations, we believe, reveal a biphasic attachment phenomenon involving an initial nonspecific, reversible process followed by a specific, irreversible attachment event.

In an attempt to support the contention that the B. subtilis

attachment represents a reversible event, the attachment kinetics of wild-type 109D to B. subtilis were followed, and the detachments were revealed by inverting each washed filter pad over a second sterile filter assembly and washing off the entrapped cells with a volume (50 ml) of x1 YPS. This suspension was stored at ambient temperature for 1 h, after which time 1 ml was refiltered and washed with 9 ml of x1 YPS and the resultant filtrate PFUs were titered (Figure 12). The initial value of the detachment curve is not the theoretically desirable zero, but is a more elevated value representing the degree of attachment advanced before the withdrawal of the first sample. Point-by-point summation of the attachment and detachment curve can be seen to yield an essentially constant value. Thus, the attachment levels and subsequent detachment of wild-type 109D to B. subtilis appear analogous to that of 109D153 to E. coli at the nonpermissive temperature.

Macromolecular capabilities of 109D153. Experiments were initiated to investigate the macromolecular capabilities of 109D153 during the infection cycle at the permissive and nonpermissive temperatures. RNA, DNA, and Bdellovibrio-specific enzyme synthesis were measured. S. serpens, preincubated in the presence of [^{3}H]uracil until uptake was complete or S. serpens plus extracellular [^{3}H]thymidine, were the sole source of nutrients. The MOI used was sufficiently large to ensure complete infection of all hosts (Table I).

During the first 5 min of infection in the presence of [^{3}H]-uracil, the net increase in trichloroacetic acid-precipitable counts

Figure 12. Attachment kinetics of wild-type 109D to Bacillus subtilis at 38.5C.

When the B. subtilis and attached Bdellovibrio cell population for each point in the attachment kinetics curve are suspended in a large volume and allowed to stand at room temperature for 1 h and the number of unattached Bdellovibrio cells is then determined, the resultant detachment curve is demonstrated. Point-by-point summation of these two curves yields a relatively constant value. The points presented represent the average of three experiments.

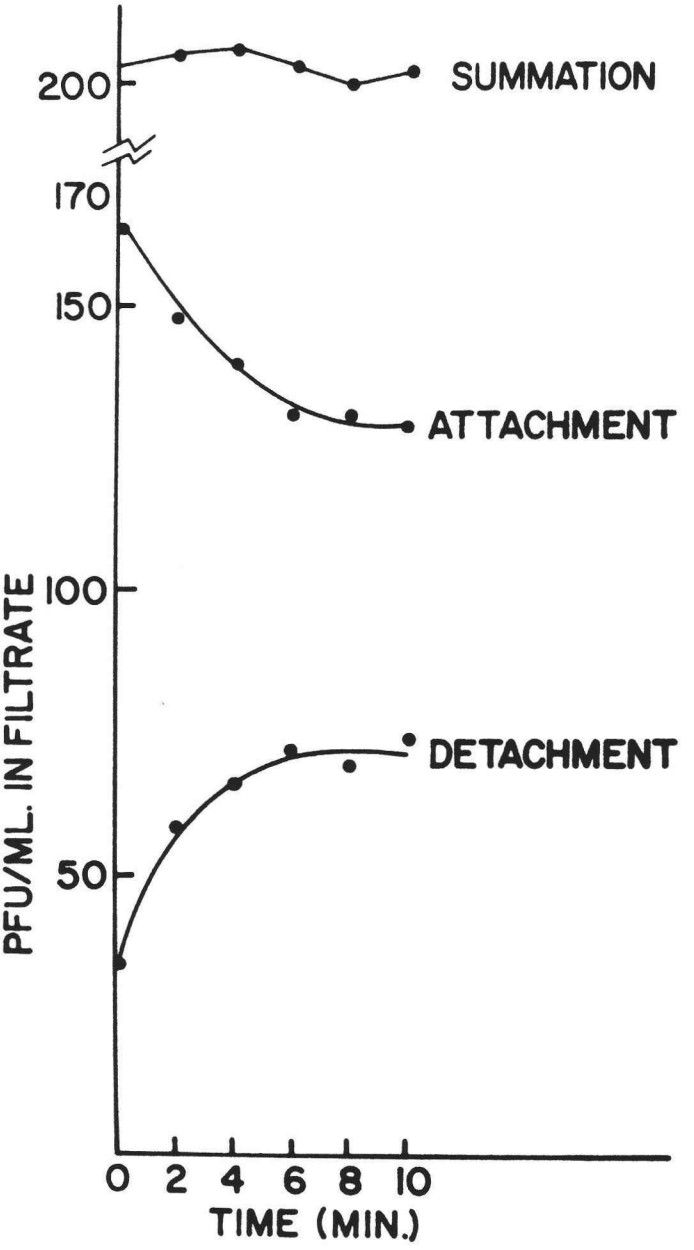

200
170
150
100
50
PFU/ML. IN FILTRATE
SUMMATION
ATTACHMENT
DETACHMENT
0 2 4 6 8 10
TIME (MIN.)

TABLE I. MACROMOLECULAR CAPABILITIES OF BDELLOVIBRIO BACTERIOVORUS 109D153 AT THE PERMISSIVE AND THE NONPERMISSIVE TEMPERATURES.

Macromolecular capabilities of H-D B. bacteriovorus 109D153 at the permissive (28 C) and the nonpermissive (38.5 C) temperatures[a]

Time	RNA				DNA (counts/min)		Protease (U/ml)	
	28 C		38.5 C					
	Counts/min	Net Counts/min	Counts/min	Net counts/min	28 C	38.5 C	28 C	38.5 C
Preincubated host	648	—	1,701	—				
Minutes after 109D153 addition								
0	522	0	1,562	0				
5	677	155	1,734	172				
10	775	253	1,540	0				
20	1,060	538	1,565	0				
30	1,338	816	1,522	0				
40	1,679	1,157	1,592	0				
Hours								
0							0.2	0.2
1					227	0		
2							0.3	0.2
4							6.8	0.2
6					9,764	21	13.7	0.2

[a] RNA values represent the incorporation of exogenous [^{3}H]uracil into cold 5% trichloroacetic acid-precipitable counts, whereas DNA values represent the incorporation of exogenous [^{3}H]thymidine into 5% trichloroacetic acid-precipitable counts. Protease assays represent the Azocollase activity of extracellular supernatant solutions. See Materials and Methods for a more complete description of techniques.

at both the permissive and nonpermissive temperatures were identical. At later time intervals, the restrictive counts either dropped to the preincubation level or shut off at the 5 min level. Permissive temperature trichloroacetic acid-precipitable counts continued to rise. These isotope incorporation studies were performed by Gwenyth G. Windom.

Mutant 109D153 failed to incorporate any [^{3}H]thymidine at the nonpermissive temperature, whereas incorporation at the permissive temperature was normal. Similarly, Bdellovibrio-specific proteases were not induced at the nonpermissive temperature, whereas at the permissive temperature induction of this proteolytic capacity followed wild-type kinetics. Protease assays were performed by Brian Klubeck.

Study of Bdellovibrio bacteriovorus HD109D intracellular growth via density-shift. In order to initiate the proposed DNA density-shift studies on Bdellovibrio bacteriovorus 109D, it was first necessary to investigate the cesium chloride equilibrium density banding patterns which could be obtained using density-labeled DNA under similar conditions. Bromouracil (5BU) substituted Escherichia coli were chosen as the logical source of trial density-labeled DNA and investigations into the preparation of 5BU substituted E. coli were undertaken. Such 5BU substituted E. coli cells were also selected as the host of choice in later B. bacteriovorus density-shift experiments.

Preparation of 5BU substituted E. coli. A thymine auxotrophic

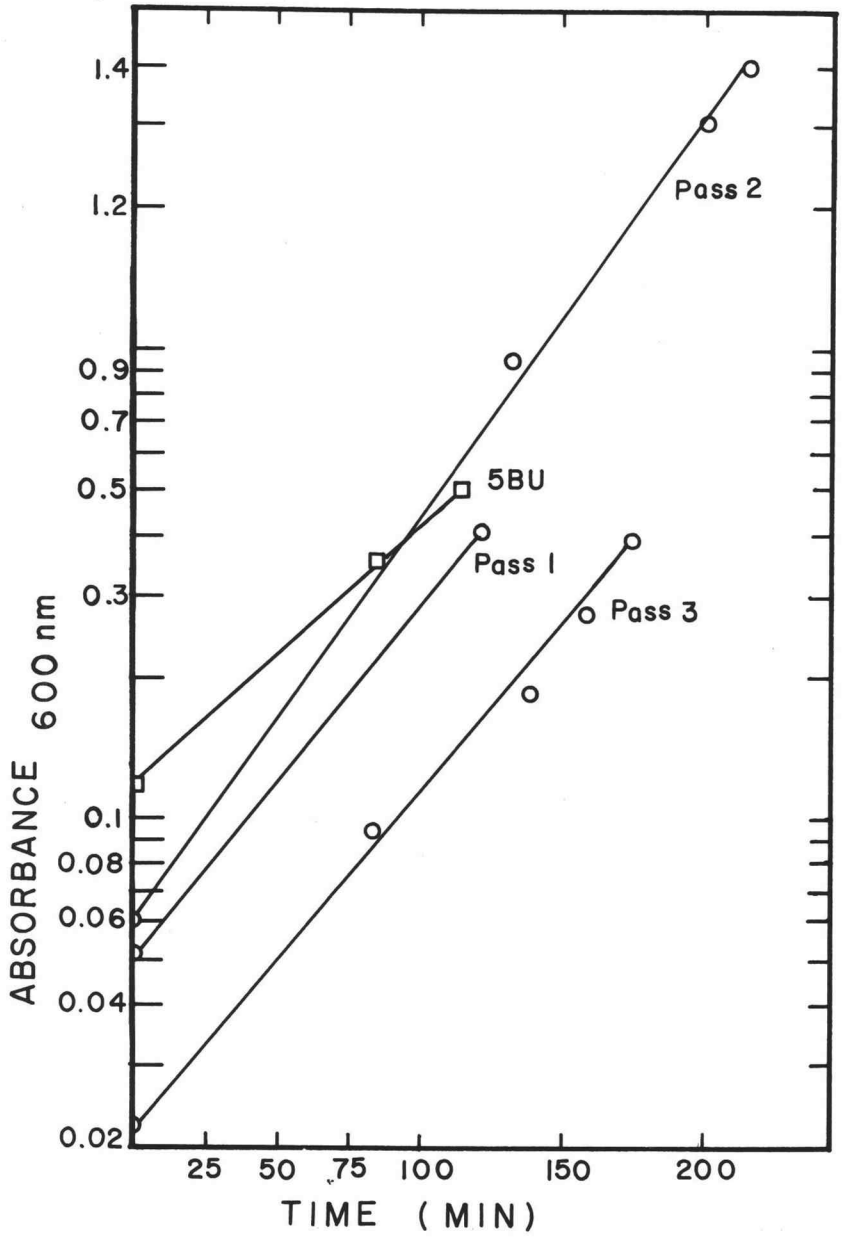
strain of E. coli (strain UC12, described in Materials and Methods) was obtained and substituted with the thymine analogue, 5-bromouracil. 5-Bromouracil substituted E. coli UC12 were prepared by the method of Hanawalt (28). The 5BU substitution regime is described in Materials and Methods. The growth response of E. coli UC12 through three transfers of the thymine supplemented minimal medium and during the substitution period in 5BU supplemented minimal medium is represented in Figure 13. When the growth rate of E. coli UC12 is determined from the experimental data contained in Figure 13, it is seen to have an average generation time of nearly 45 min (or 1.3 generations/h) in the thymidine supplemented minimal medium used. This growth rate slows to a generation time of 65 min (or 0.9 generations/h) over the 5BU substitution period.

Hanawalt and Ray (29) have noted that at 37C the DNA synthesis rate of E. coli in the presence of 5BU is about half that for cultures growing with thymidine. Moreover, during growth in 5BU containing medium, E. coli demonstrates an inhibition of cell division (28) and hence tends to grow progressively longer until after extended periods in the presence of 5BU, long filamentous forms are observed. This cell elongation can serve as an in vivo index of the onset of 5BU substitution.

To determine what degree of elongation was occurring under our conditions of 5BU substitution, an uncalibrated microscope ocular micrometer was used to follow changes in cell size (in abitrary units) with time. Fifty cells were measured at various time periods after

Figure 13. Growth response of Escherichia coli UC12 to the presence of 5BU.

The increase in A_{600} of E. coli UC12 during three passages in thymine (2 µg/ml) supplemented minimal medium (o) and one passage in 5BU (10 µg/ml) supplemented minimal medium (□) is illustrated. For further details see Materials and Methods.


ABSORBANCE 600 nm
1.4
1.2
0.9
0.7
0.5
0.3
0.1
0.08
0.06
0.04
0.02
25
50
75
100
150
200
TIME (MIN)
Pass 2
5BU
Pass 1
Pass 3

the transfer of E. coli UC12 to 5BU containing medium. The average length per cell obtained for each time period is represented in Table II. It can be seen that after 120 min exposure to 5BU under the conditions tested, the average cell length in an E. coli UC12 cell population has more than doubled. Though not shown in Table II, E. coli UC12 cell length at 180 min after exposure to 5BU was greater than four times normal, with some individual cells being greater than 50 μm in length. One hundred twenty minutes exposure of E. coli UC12 to 5BU was chosen for the substitution period to be used in later experiments. Thus, the phrase "5BU substituted E. coli" as used in the remainder of this text will refer to E. coli UC12 density-labeled for 120 min under the conditions described.

Conditions for CsCl equilibrium density gradient centrifugation. To investigate the cesium chloride equilibrium density profiles and banding patterns which could be expected using the Type 50 rotor under the conditions planned, centrifuge tubes containing highly purified Bdellovibrio bacteriovorus H-I 109D DNA and highly purified Escherichia coli strain UC12 5BU substituted DNA were prepared in 5 ml CsCl solutions of various initial mean densities. Following centrifugation at 40,000 rev/min for 48 h at 26C, fractions were collected and their absorbance at 260 nm determined. The results are represented in Figures 14-16. As can be seen, adequate resolution of B. bacteriovorus H-I 109D DNA and the light/heavy fraction of 5BU substituted E. coli UC12 DNA are achieved at all the initial CsCl densities tested. However, it is only at the greater initial

TABLE II. EFFECT OF 5-BROMOURACIL LABELING UPON *ESCHERICHIA COLI* UC12 CELL LENGTH.

TIME (MIN)	AVERAGE CELL LENGTH[a]
0	5
60	5.4
90	8.1
120	11.5

[a] *E. coli* UC12 was grown as described (Materials and Methods, page 24) in the presence of 10 µg/ml 5BU. At the times indicated, 50 cells were measured in arbitrary units using an uncalibrated microscope ocular micrometer.

Figure 14. Separation of H-I Bdellovibrio bacteriovorus 109D and 5BU substituted Escherichia coli UC12 DNA following CsCl equilibrium density gradient centrifugation.

0.2 mg each of highly purified H-I B. bacteriovorus 109D and 5BU substituted E. coli UC12 DNA in 0.1 X SSC (pH 7.5) were mixed with solid CsCl and submitted to equilibrium density gradient centrifugation at 26C in a type 50 rotor at 40,000 rpm. Fractions were collected from the bottom and the A_{260} of each fraction determined. The initial mean densities were adjusted to 1.72 g/ml (upper figure) and 1.73 g/ml (lower figure).

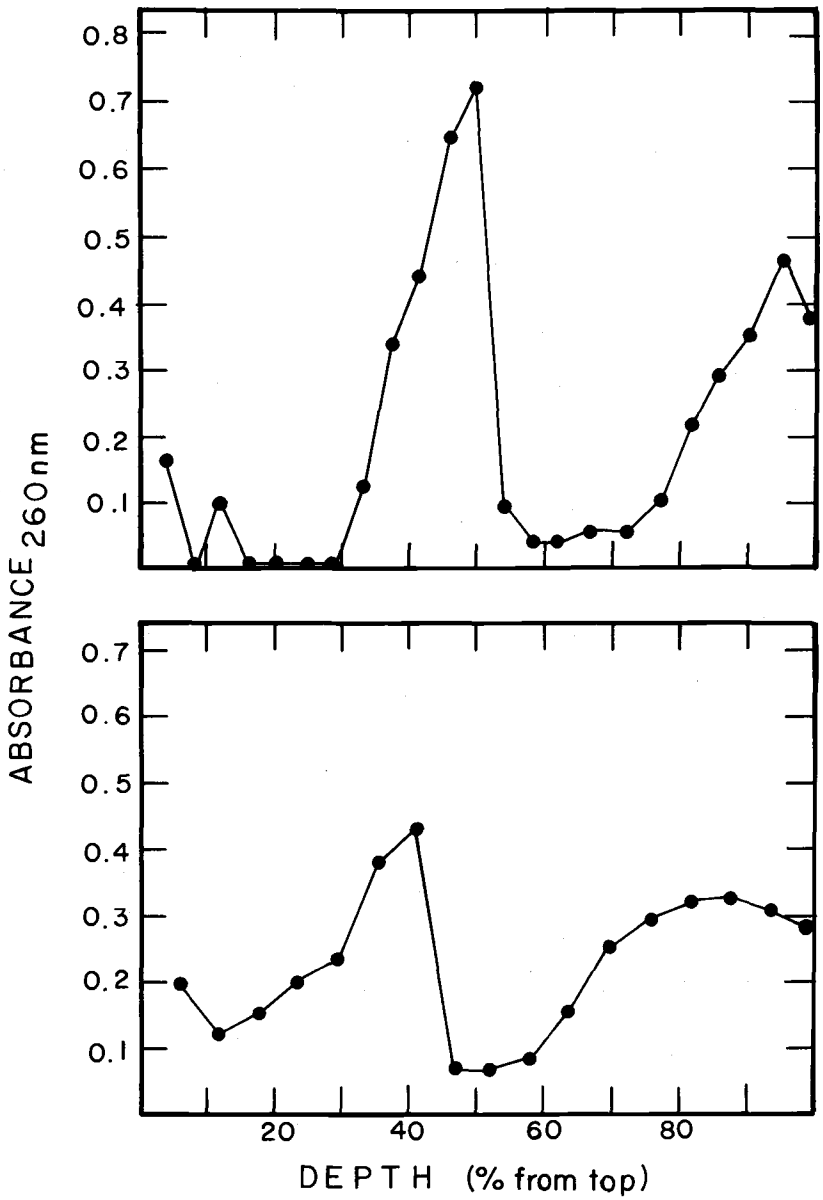

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE 260nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
DEPTH (% from top)

Figure 15. Separation of H-I Bdellovibrio bacteriovorus 109D and 5BU substituted E. coli UCl2 DNA following CsCl equilibrium density gradient centrifugation.

As in Figure 14. Initial mean density was 1.74 g/ml. Lower figure illustrates the density profile of a blank gradient adjusted to an initial mean CsCl density of 1.738 g/ml.

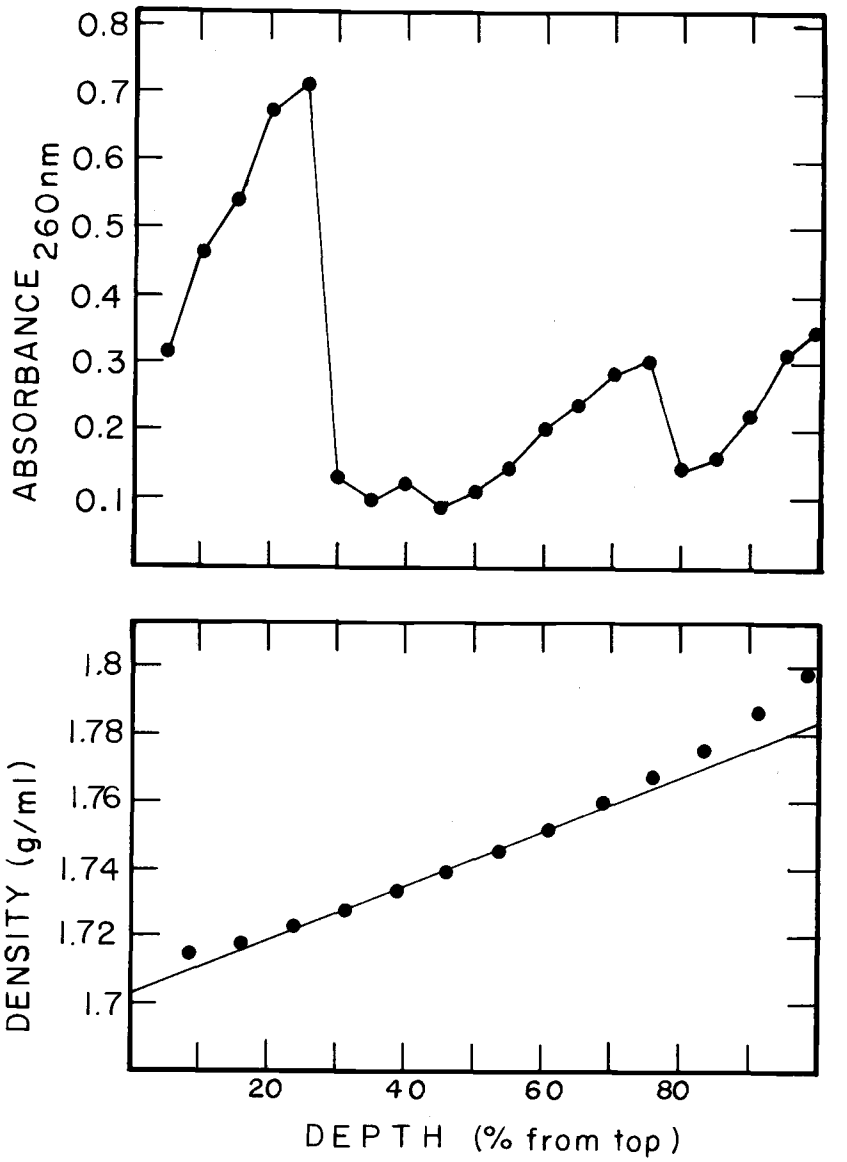
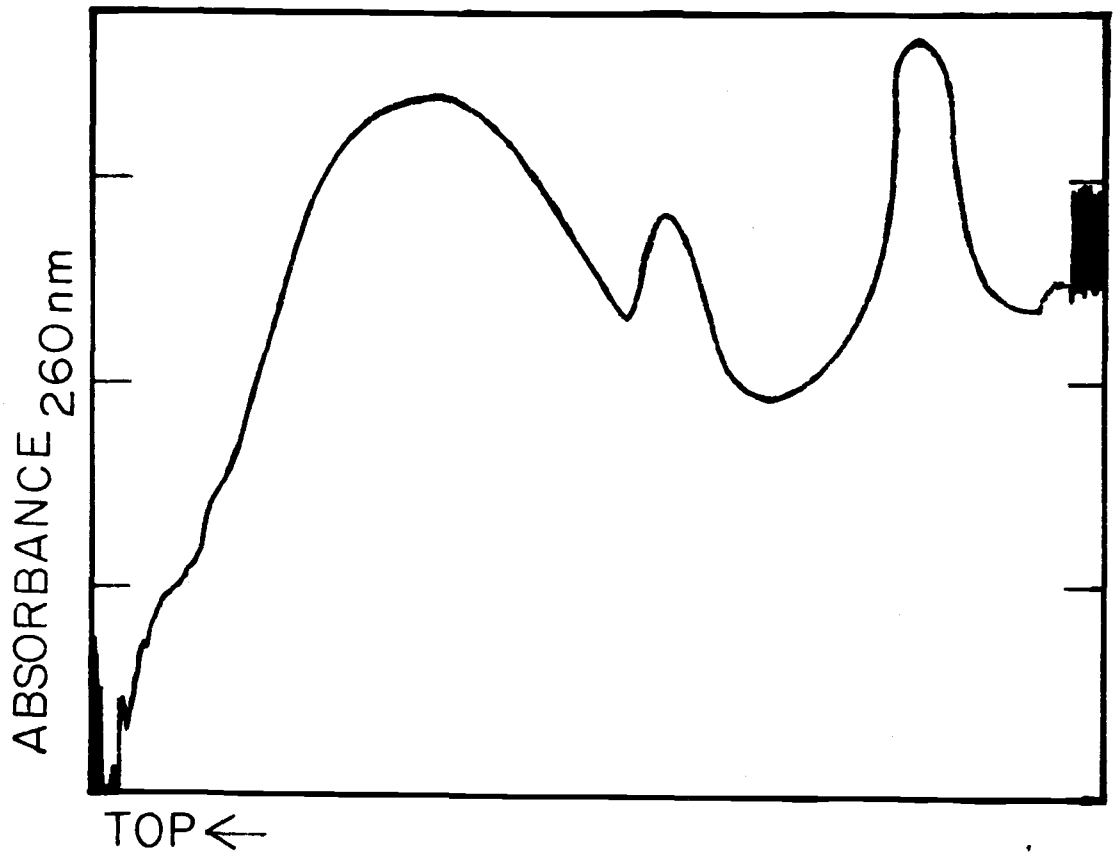

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE 260nm
1.8
1.78
1.76
1.74
1.72
1.7
DENSITY (g/ml)
20
40
60
80
DEPTH (% from top)

Figure 16. Separation of H-I Bdellovibrio bacteriovorus 109D and 5BU substituted E. coli UC12 DNA following CsCl equilibrium density gradient centrifugation.

As in Figure 14, except the gradient was collected from the top and pumped through a flow-through cuvette mounted in a Gilford 2000 Recording Spectrophotometer. Full scale deflection was set at one optical density unit. Initial mean density of gradient was adjusted to 1.735 g/ml. DNA samples were sheared by two passages through a Carver French Press at 14,000-16,000 psi. 0.275 mg B. bacteriovorus and 0.53 mg of E. coli UC12 5BU substituted DNA were run.


ABSORBANCE 260nm
TOP

CsCl densities that the heavy/heavy fraction of 5BU substituted E. coli UC12 DNA are buoyed sufficiently to prevent their being pelleted into the bottom of the centrifuge tube.

B. bacteriovorus H-I 109D DNA has a T_m and moles percent G+C content nearly equal to that of E. coli DNA and would be expected to band at a solution density of 1.71 g/ml as does E. coli DNA. 5BU substituted E. coli UC12 could be expected to yield DNA fragments banding at unsubstituted, one strand substituted and both strands substituted positions in a CsCl equilibrium density gradient. One strand substituted (light/heavy) E. coli DNA should band at a CsCl density of greater than 1.71 g/ml, depending upon the degree of substitution of 5BU for thymine. E. coli TAU-bar 5BU light/heavy hybrid DNA prepared by the same method used in the preparation of 5BU substituted E. coli UC12 has a density of 1.756 g/ml. E. coli TAU-bar 5BU heavy/heavy DNA has a density of greater than 1.8 g/ml.

The precise banding densities of 5BU substituted DNA in gradients like those illustrated in Figures 14-16 is difficult to determine. DNA in CsCl solution interferes with attempts to determine the CsCl density via refractive index readings by shifting such readings to lesser values. Refractive index readings may be taken on gradient fractions before and after the light/heavy 5BU substituted E. coli UC12 DNA peak. Gradient density profiles as in Figure 15 (lower) may be generated via refractive index readings on fractions of a blank gradient made and centrifuged so as to be nearly identical to an experimental gradient. By these two techniques, 5BU substituted E. coli UC12 DNA prepared as described bands at 1.76 g/ml (light/heavy)

and 1.81 g/ml (heavy/heavy).

Figure 16 represents the 250 nm absorbance tracing obtained if a gradient (initial mean density: 1.735 g/ml) containing 275 µg of highly purified, sheared (Carver French Press, two passes at 14,000-16,000 psi) B. bacteriovorus H-I 109D DNA and 530 µg of highly purified E. coli UC12 5BU substituted DNA is collected from the top following centrifugation and passed through a 0.25 ml flow-through cuvette in a Gilford 2000 recording spectrophotometer. Full scale deflection was calibrated to equal 1.0 O.D. unit. The high preparative loading capacity of angle rotor gradients is quite graphically demonstrated. This high loading capacity would be quite important for the preparation by CsCl equilibrium density gradient centrifugation of large quantities of DNA, such as the milligram quantities of DNA needed to perform DNA renaturation kinetics determinations by optical means.

Infection of 5BU substituted E. coli with isotopically labeled Bdellovibrio bacteriovorus 109D. To follow DNA replication during the intracellular growth phase of Bdellovibrio bacteriovorus H-D 109D, 5BU substituted E. coli UC12 cells were infected under one-step growth conditions. Isotopically labeled Bdellovibrio cells were prepared as described (Materials and Methods). The 25,500 x g Bdellovibrio pellet was resuspended in PYE/10 to an A_{600} of 1.5 and used to infect 5BU substituted E. coli UC12 (prepared as described and stored on ice until use, not more than four h) resuspended in PYE/10 to an A_{600} of 30.0. One part of each cell suspension was

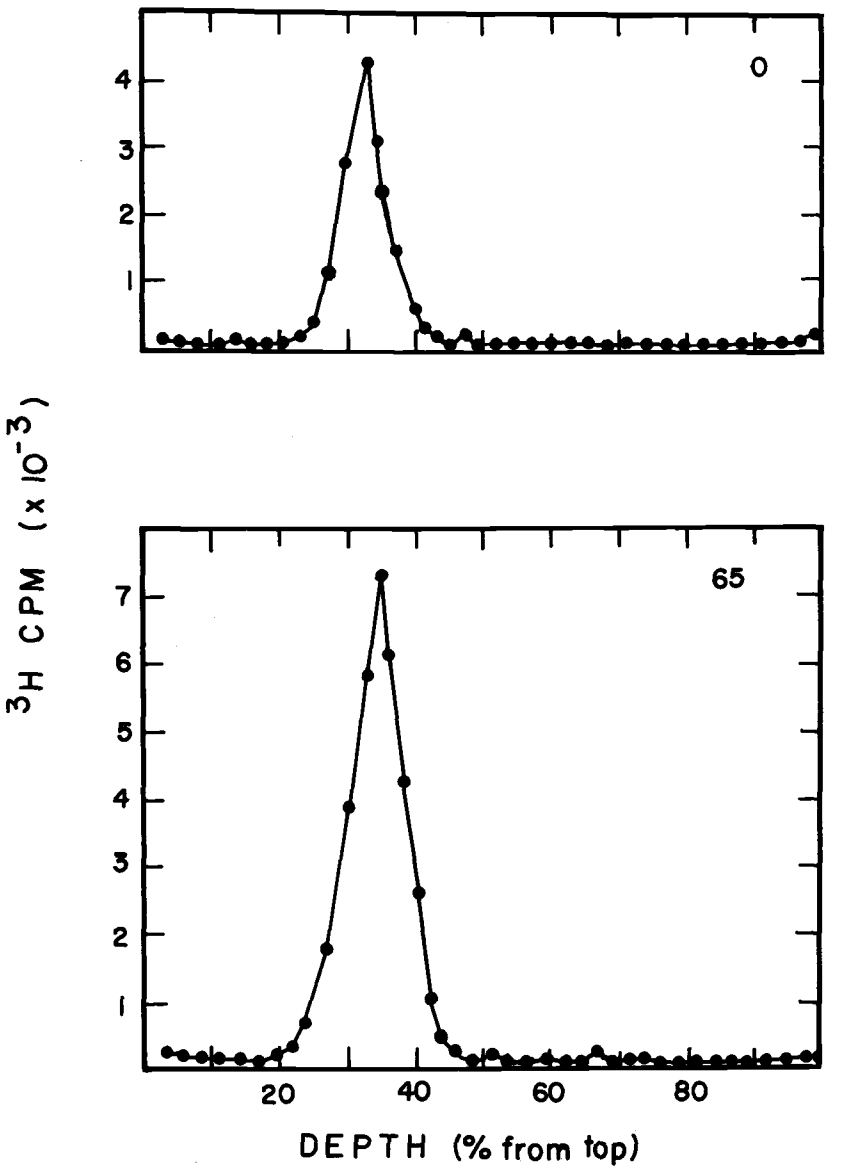
added to eight parts prewarmed PYE/10 and shaken at 30C in the dark. At various times thereafter, growth was stopped by the addition of an equal volume of chilled killing solution and cells were harvested by centrifugation at 12,000 x g for 20 min. The cell pellets obtained were frozen, subsequently thawed, and nucleic acids extracted as described (Materials and Methods). The resulting CsCl equilibrium density profiles obtained following centrifugation of extracted nucleic acids from several such experiments are presented in Figures 17-25.

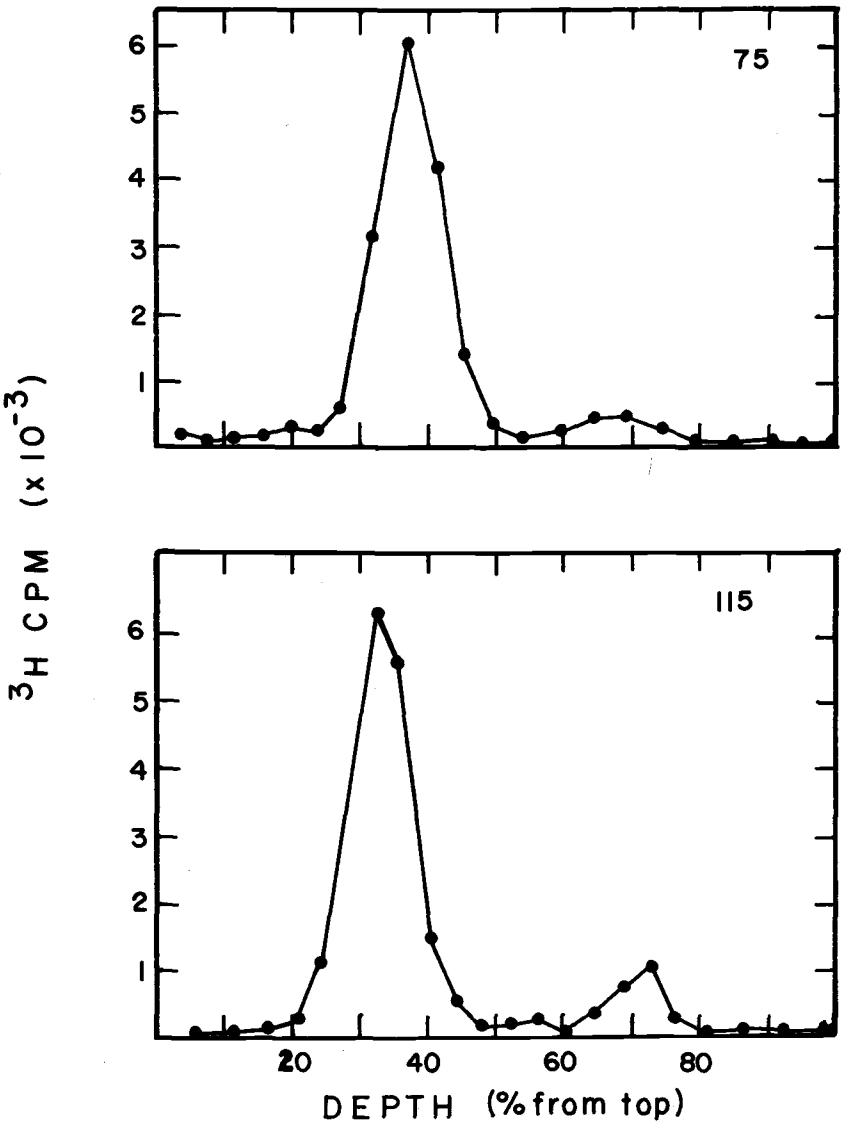
The results demonstrate the presence of an isotopically labeled, density shifted, ^{3}H-thymidine containing peak which is not present at zero time (Figure 17, upper) or 65 min postinfection (Figure 17, lower); has begun to accumulate by 75 min (Figure 18, upper); and by 85 min has reached a maximum intensity which is maintained thereafter (Figures 20-25, Appendix). The sum of the counts in the hybrid peak represents an average of 18.8% of the sum of the counts in the light peak.

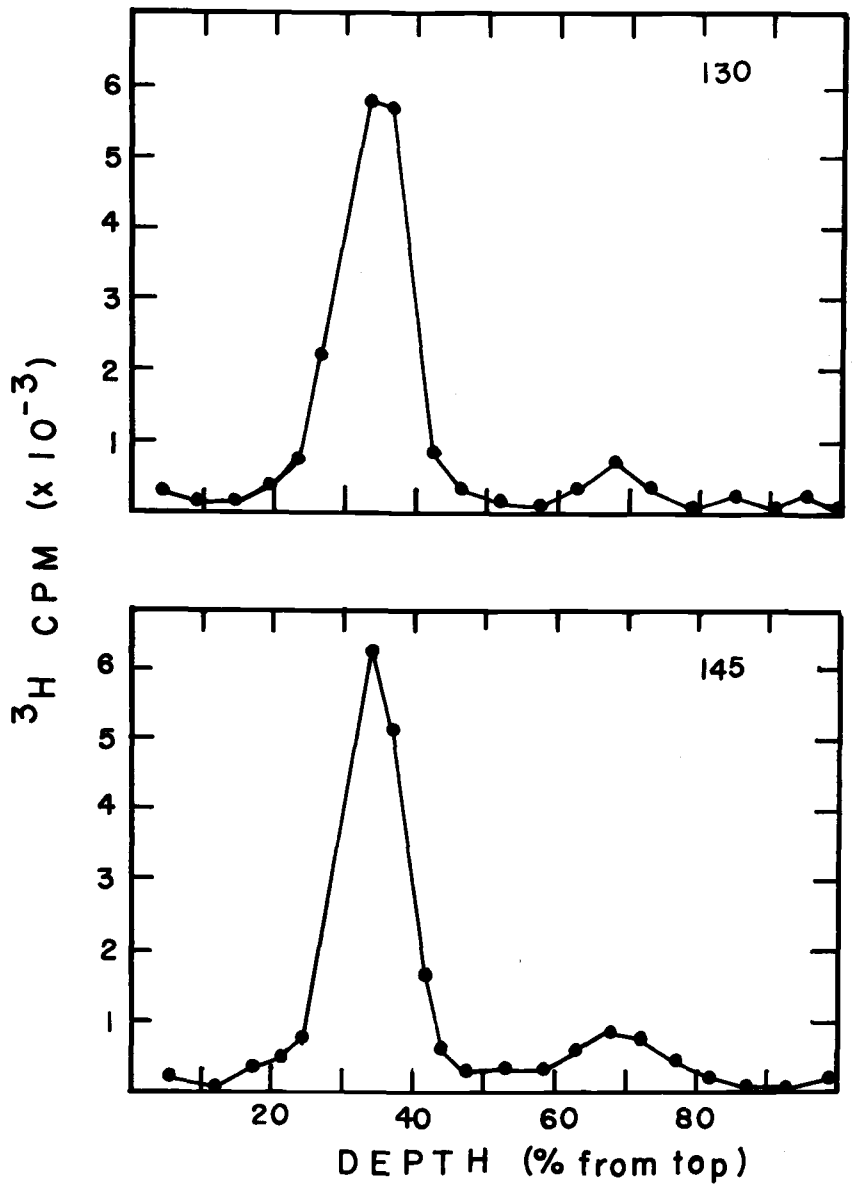
It is important to note that although the appearance of this peak begins relatively early (75 min), its maximum intensity is attained rapidly (85-90 min). This is not to say that further production is blocked, only that a maximum accumulation is reached. For it must be noted that in this system, conventional semi-conservative replication of isotopically labeled parental strands would yield a light/heavy hybrid molecule of specific activity reduced proportionally by one-half times the degree of substitution and a heavy/heavy molecule of

Figures 17-25. CsCl equilibrium density gradient analysis of the infection of 5BU substituted E. coli UC12 with Bdellovibrio bacteriovorus 109D.

5BU substituted E. coli UC12 were infected under one-step growth conditions with isotopically labeled Bdellovibrio. At the times indicated following infection, samples were mixed with an equal volume of a chilled saline-EDTA-KCN killing solution, centrifuged and the cell pellets frozen at -20C. Subsequently the samples were thawed and the DNA extracted and submitted to CsCl equilibrium density gradient centrifugation. Fractions were collected from the top and the TCA precipitable counts determined. For further details see Materials and Methods, pages 25-28. The results of three separate experiments are presented. For convenience the results of one experiment (Figures 17-19) are presented here (pages 62a-c), while the remainder (Figures 20-25) are presented in an appendix (page 78).


0
65
4
3
2
1
7
6
5
4
3
2
1
20
40
60
80
^{3}H CPM (x 10^{-3})
DEPTH (% from top)


75
115
^{3}H CPM (x 10^{-3})
1
2
3
4
5
6
20
40
60
80
DEPTH (% from top)


130
145
^{3}H CPM (x 10^{-3})
1
2
3
4
5
6
20
40
60
80
DEPTH (% from top)

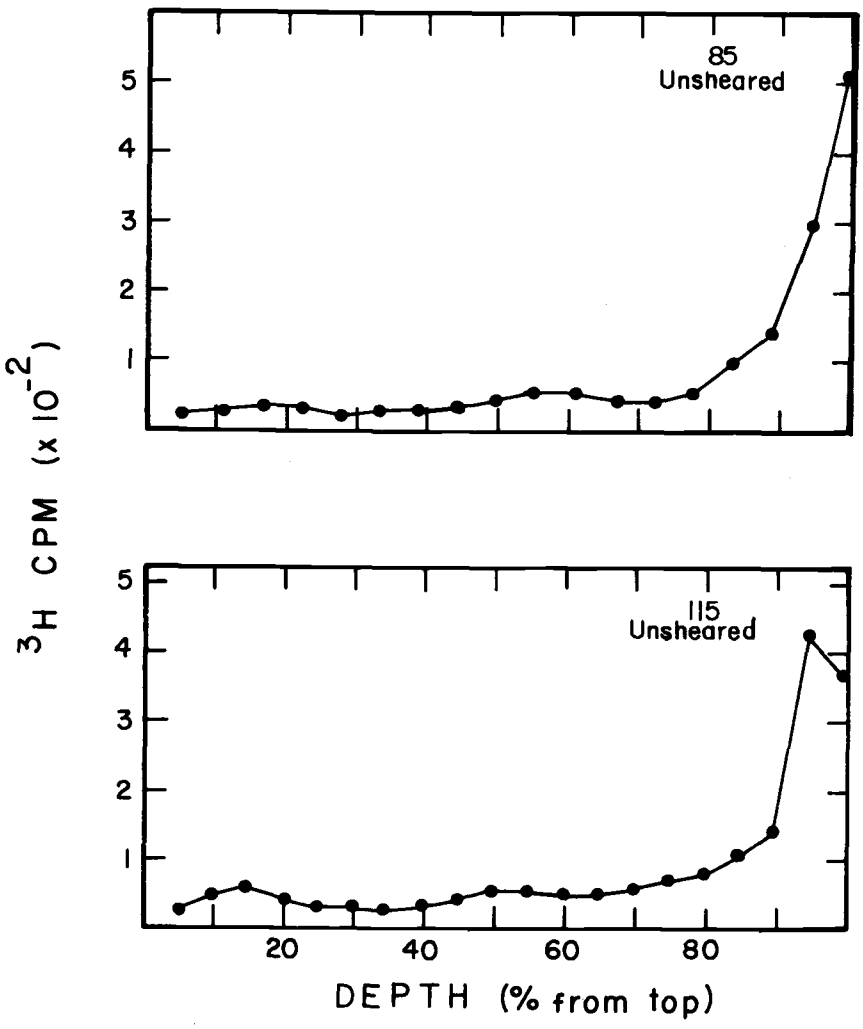
specific activity reduced proportionally by the degree of substitution of 5BU for thymine. Thus, any heavy/heavy molecules produced may have specific activities so reduced as to be detectable only in relatively large amounts. However, it must also be noted that even as late as 175 min after infection, or 90 min after the maximum hybrid level is attained, the size of the light peak is virtually undiminished. This observation argues against the possibility that the hybrid peak maintainance demonstrated represents an equilibrium state between conversion of light to hybrid and hybrid to heavy/heavy.

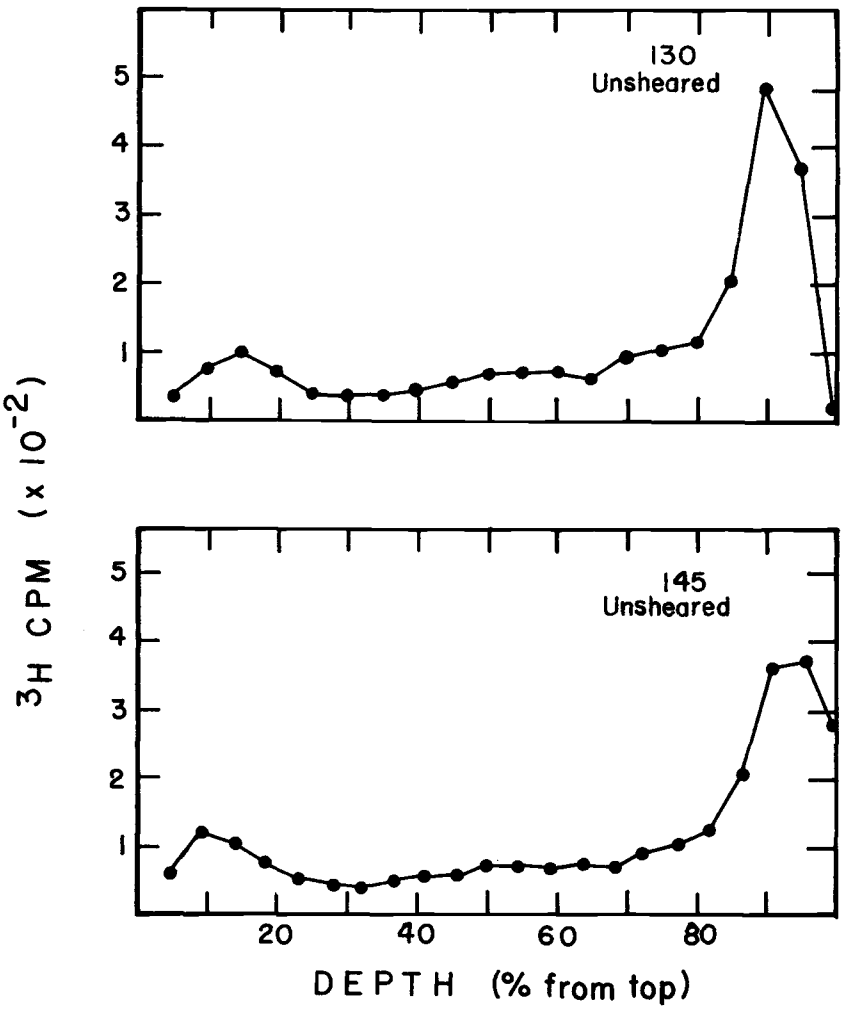
Infection of ^{3}H-5BU substituted E. coli with Bdellovibrio bacteriovorus 109D. To gain more information on the nature of the chromosomal events occurring in this system, 6-^{3}H-5BU substituted E. coli UC12 were prepared and infected by unlabeled Bdellovibrio under conditions allowing for one-step growth. 5BU-substituted E. coli UC12 were prepared as described with the exception that 5 µg/ml of 5BU and 1 µCi/ml of 6-^{3}H-5BU (23 Ci/mM) were used to supplement the minimal medium during the 5BU-incorporation period rather than the 10 µg/ml of 5BU used previously. Figures 26-28 present the isotope distribution patterns obtained following CsCl equilibrium density centrifugation of nucleic acids extracted as described from these interrupted one-steps.

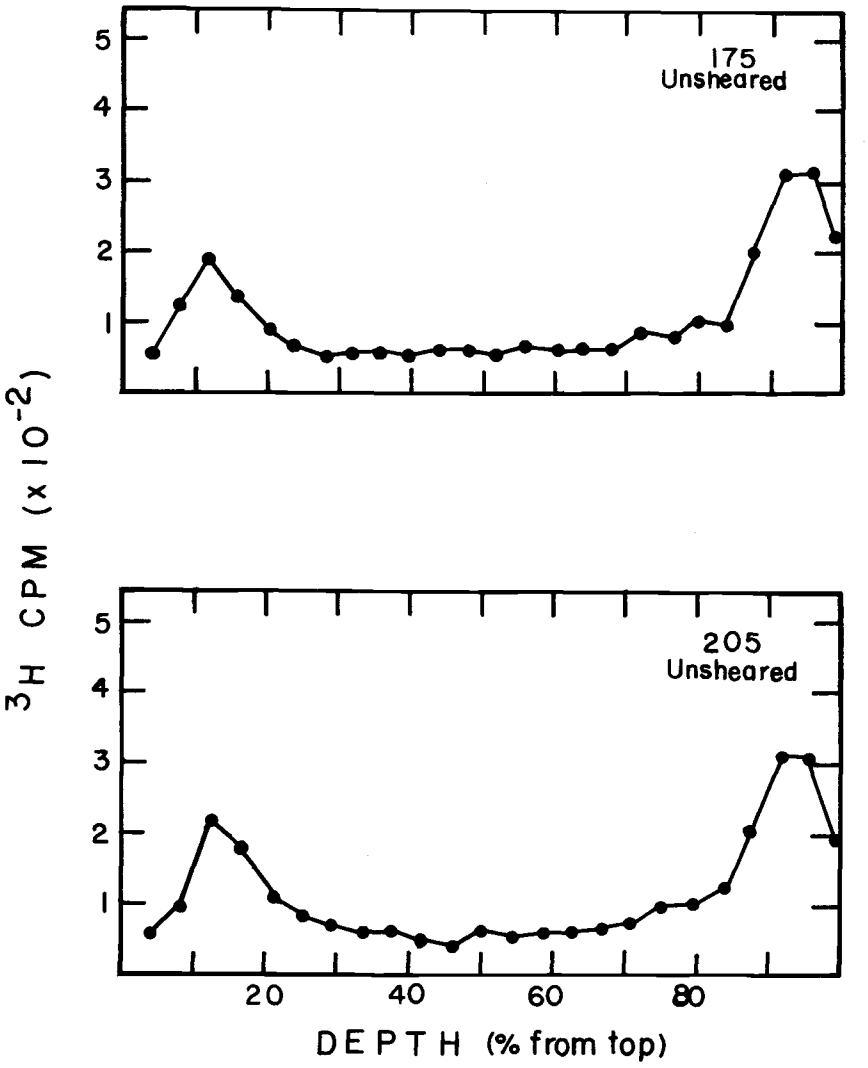
Together these profiles demonstrate a continuous loss with time of counts banding in the lower portion of the gradients and the emergence of 6-^{3}H-5BU counts in the light position of the gradients. The detection of any newly formed hybrid DNA species (expected here

Figures 26-28. CsCl equilibrium density gradient analysis of the infection of 6-^{3}H-5BU substituted E. coli UC12 with Bdellovibrio bacteriovorus 109D.

5BU substituted E. coli UC12 were prepared as described (Materials and Methods) with the exception that 5 µg/ml of 5BU and 1 µC/ml of 6-^{3}H-5BU replaced the 10 µg/ml 5BU initially used. At the times indicated following the infection of these isotopically labeled, 5BU substituted E. coli with B. bacteriovorus 109D, samples were mixed with an equal volume of a chilled saline-EDTA-KCN killing solution, centrifuged and frozen at -20C. Subsequently the samples were thawed and the DNA extracted and submitted to CsCl equilibrium density gradient centrifugation. Fractions were collected from the top and TCA precipitable counts determined.


85
Unsheared
115
Unsheared
5
4
3
2
1
20
40
60
80
^{3}H CPM (x 10^{-2})
DEPTH (% from top)


130
Unsheared
145
Unsheared
5
4
3
2
1
20
40
60
80
DEPTH (% from top)
^{3}H CPM (x 10^{-2})


175
Unsheared
205
Unsheared
^{3}H CPM (x 10^{-2})
DEPTH (% from top)
1
2
3
4
5
20
40
60
80

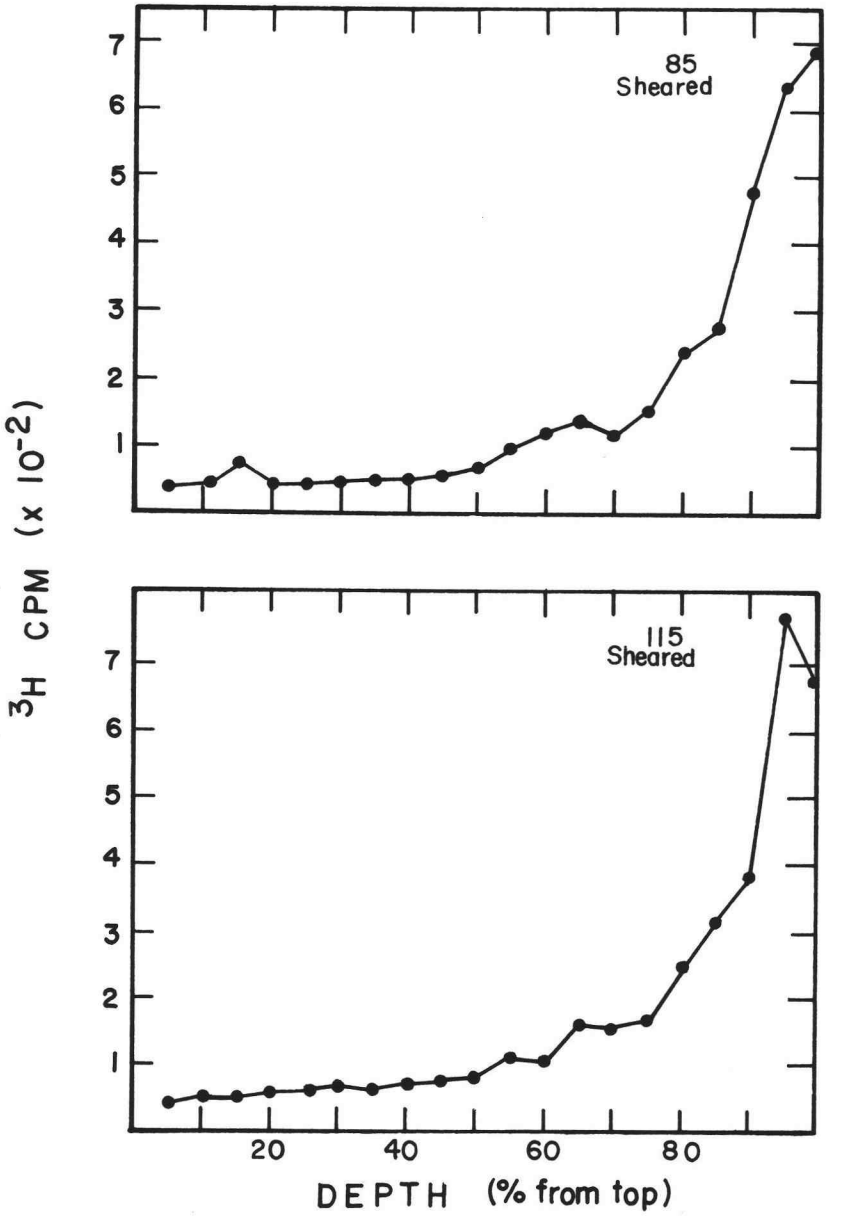
to fall between 65% and 80% down in the gradients) is made difficult due to the fragmentation and smearing of host DNA apparently taking place. The ratio of the total counts in the top 50% of the 205 min gradient and the total counts in the bottom 50% of the same gradient is 2/3.

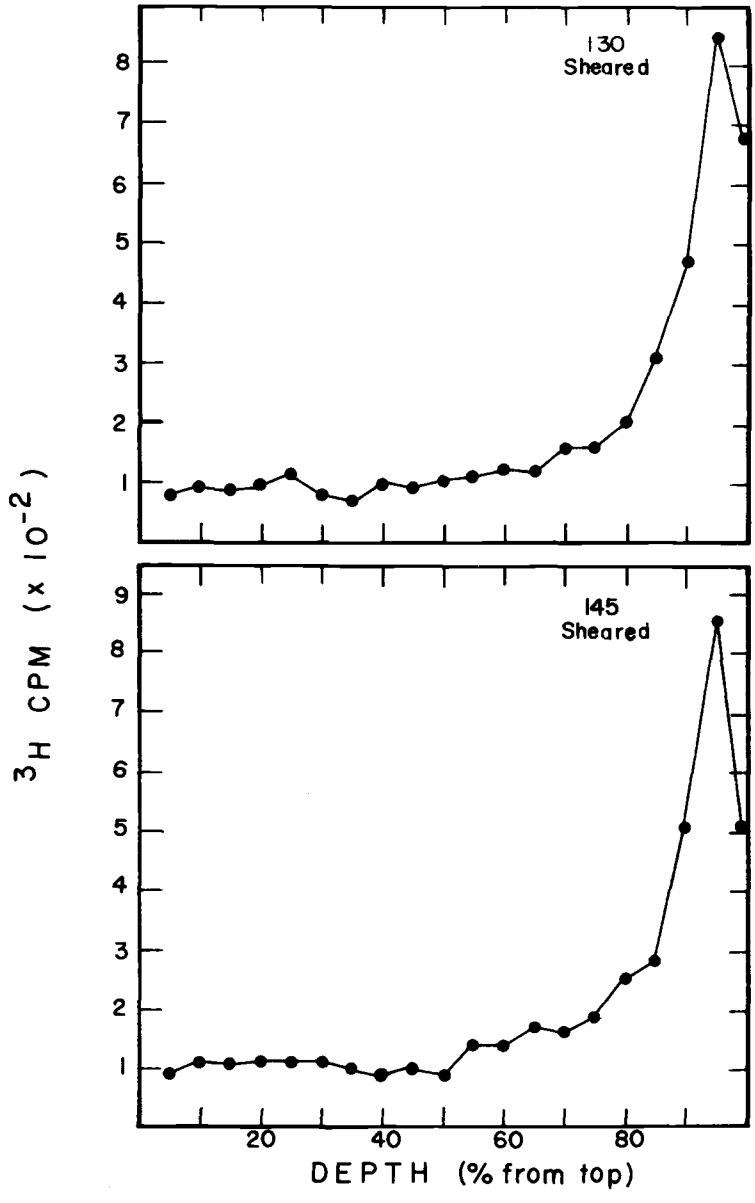
When the same nucleic acid samples seen in Figures 26-28 are sheared by two passes through a Carver French Press at 14,000-16,000 psi and then subjected to CsCl equilibrium density centrifugation, the isotope distribution profiles illustrated in Figures 29-31 are observed. Comparing Figures 26-28 with Figures 29-31, the general pattern which emerges is that shearing the samples prior to centrifugation produces a decrease in the number of counts banding in the upper portions of the gradients. To confirm this observation, unsheared samples were subjected to CsCl equilibrium density centrifugation (as in Figures 26-28) and then the upper 30% of each gradient was pooled, sheared in the French Press and then rebanded. The isotope distribution profiles illustrated in Figures 32-34 demonstrate a shear induced release of counts from the top portion of the gradients to the lower portions of the gradients. However, they also demonstrate a large accumulation of ^{3}H-5BU in the upper portions of the gradients which is not released to the lower gradient portions by shearing.

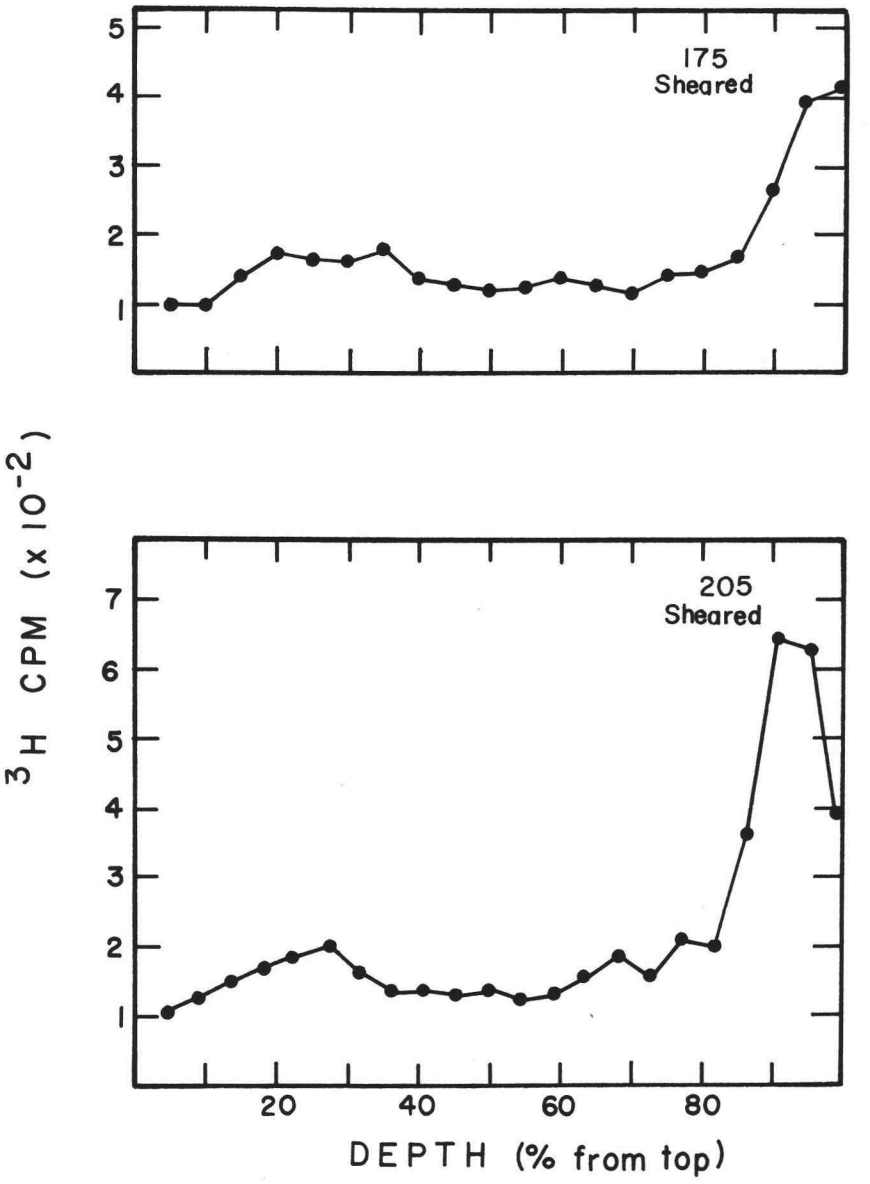
Table III, which represents the proportion of the total counts in each gradient of Figures 26-34 which band in the upper 50% of the gradient and the proportion which band in the lower 50%, summarizes

Figures 29-31. CsCl equilibrium density gradient analysis of the infection of 6-^{3}H-5BU substituted *E. coli* UC12 with *Bdellovibrio bacteriovorus* 109D.

As in Figures 26-28, with the exception that all DNA samples were sheared by passage two times through a Carver French Press at 14,000-16,000 psi prior to density gradient analysis.

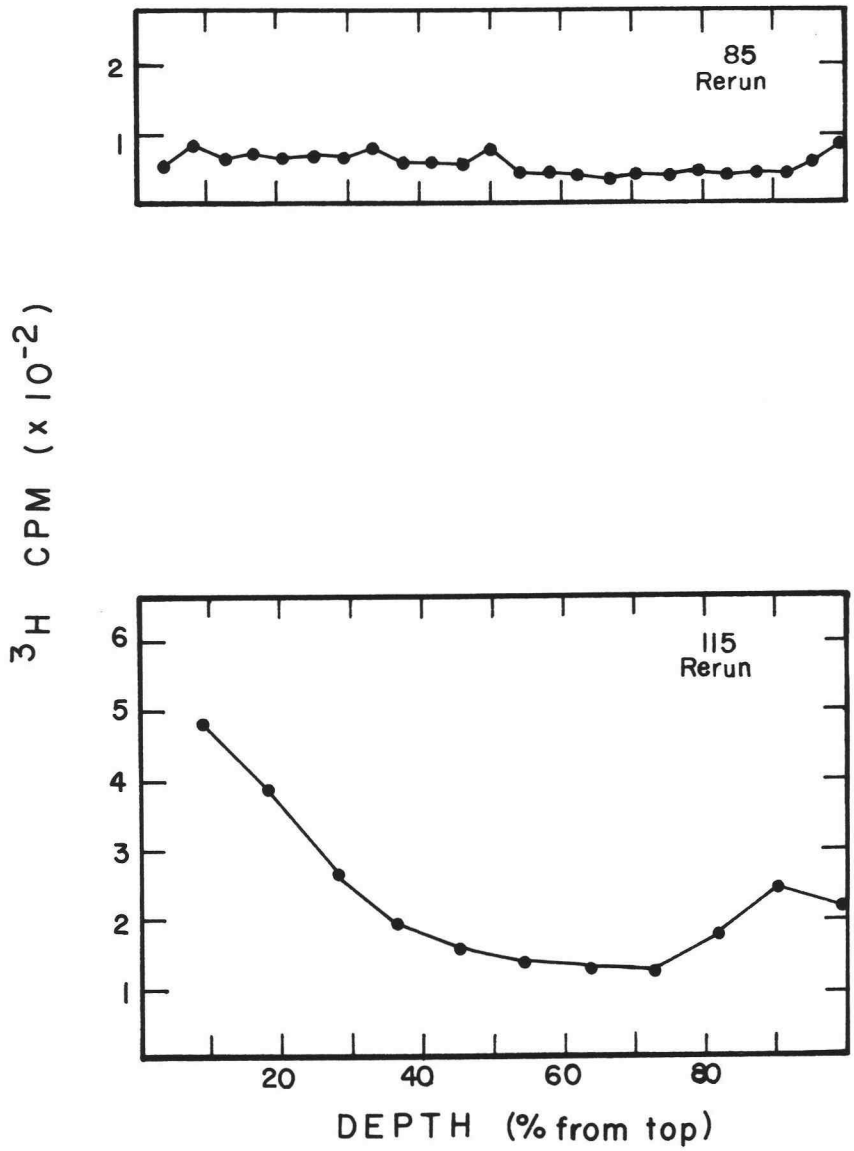

85
Sheared
115
Sheared
^{3}H CPM (x 10^{-2})
DEPTH (% from top)
1
2
3
4
5
6
7
20
40
60
80

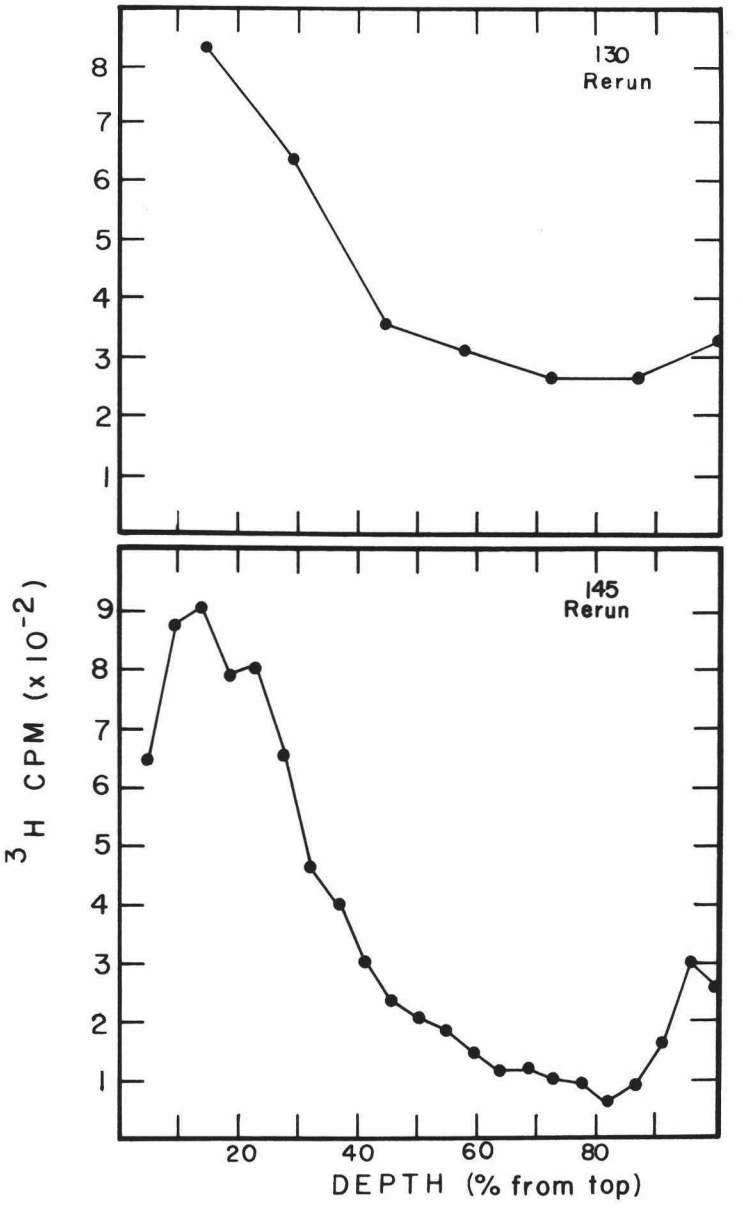

130
Sheared
145
Sheared
^{3}H CPM (x 10^{-2})
DEPTH (% from top)
20
40
60
80


175
Sheared
205
Sheared
^{3}H CPM (x 10^{-2})
DEPTH (% from top)
1
2
3
4
5
6
7
20
40
60
80

Figures 32-34. CsCl equilibrium density gradient rebanding of the upper 30% of Figures 26-28 following shearing.

As in Figures 26-28, except following CsCl equilibrium density gradient centrifugation the upper 30% of each gradient was collected, twice passaged through a Carver French Press at 14,000-16,000 psi and resubmitted to CsCl equilibrium density gradient centrifugation. Following centrifugation fractions were collected from the top and TCA precipitable counts determined.


85
Rerun
115
Rerun
^{3}H CPM (x 10^{-2})
DEPTH (% from top)
1
2
3
4
5
6
20
40
60
80


130
Rerun
145
Rerun
^{3}H CPM (x10^{-2})
DEPTH (% from top)
20
40
60
80

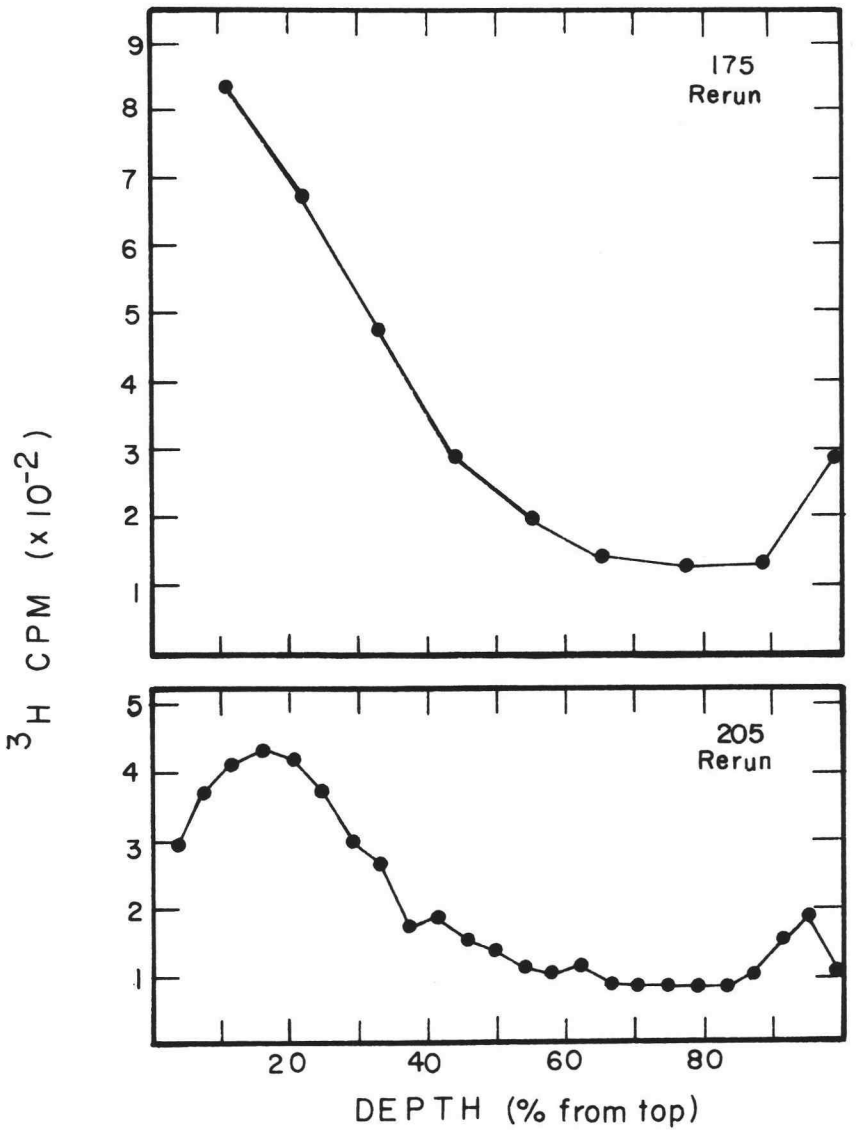

175
Rerun
205
Rerun
^{3}H CPM (x 10^{-2})
DEPTH (% from top)
20
40
60
80

TABLE III. BDELLOVIBRIO INFECTION OF ^{3}H-5BU LABELLED E. COLI

I. Nucleic acid extracted and centrifuged (Figures 20-25)

Time after infection (min)	^{3}H counts in upper 50% of gradient (% of total counts in gradient)	^{3}H counts in lower 50% of gradient (% of total counts in gradient)
85	18	82
115	23	77
130	27	73
145	29	71
175	37	63
205	40	60

II. Nucleic acid extracted, sheared, and centrifuged (Figures 26-31)

Time after infection (min)	^{3}H-5BU counts in upper 50% of gradient (% of total counts in gradient	^{3}H-5BU counts in lower 50% of gradient (% of total counts in gradient)
85	13	87
115	17	83
130	23	77
145	25	75
175	41	59
205	34	66

III. Nucleic acid extracted, centrifuged, top 30% of gradient collected, sheared, and recentrifuged (Figures 32-37)

Time after infection (min)	^{3}H-5BU counts in upper 50% of gradient (% of total counts in gradient)	^{3}H-5BU counts in lower 50% of gradient (% of total counts in gradient)
85	57	43
115	59	41
130	61	39
145	79	21
175	73	27
205	73	27

these results.

Table III illustrates an increase with time in the percentage of counts banding in the upper 50% of the unsheared gradients (Figures 26-28), a drop in this percentage following shearing prior to centrifugation (Figures 29-31), and a large accumulation of non-shear releasable counts in the upper 30% of the gradients (Figures 32-34). These observations together are interpreted as indicating a major incorporation of 5BU into Bdellovibrio DNA in a random manner.

DISCUSSION

The mutants reported upon here demonstrate reversion frequencies of 10^{-8} or 10^{-9} with the lawn overlay technique described. They also all fail to yield PFU increases when the 38.5C two-membered cultures are prepared by mixing temperature-equilibrated host and Bdellovibrio cells. Mutants were, however, isolated that met the first criterion but not the second. This class of organisms apparently represents mutants which can initiate at least partial rounds of replication before the effects of the deficiency are sufficiently felt to prevent further replication. It is to be emphasized that the low levels of PFU percent increases seen at some time periods of the temperature-shifted, one-step growth experiments probably does not represent this type of growth but is, more probably, a reflection of Bdellovibrio infection cycle asynchrony.

Penetration-deficient mutants 109D3 and 109D48 fail to multiply at the nonpermissive temperature. The manifestation of this outcome occurs early in the infection cycle as a failure of the Bdellovibrio mutant to invade the host cell periplasmic compartment. It is not possible with the data presently at hand to determine whether the failure of 109D3 and 109D48 to penetrate the host cell is directly responsible for their subsequent failure to multiply or whether their failure to penetrate is a secondary manifestation of other defects.

109D4 and 109D152 fail to demonstrate PFU increases at the non-permissive temperature although their infection cycle proceeds normally until the later stages of intracellular growth. The events

occurring during the intracellular stages of Bdellovibrio infection cycle are undoubtedly complex, and our data do not attempt to differentiate between the various intracellular developmental stages (elongation, fragmentation, and burst). Electron microscope studies are in progress on these mutants elsewhere and should help distinguish further the specific nature of the block.

The isolation of temperature-sensitive Bdellovibrio mutants that are blocked at different morphological stages of the infectious life-cycle is indicative of a physiological function unique to each stage of development. The mutants that are, morphologically, blocked early (such as 109D153) fail to initiate physiological functions normally associated with later intracellular development (DNA and protease synthesis). This observation, and the fact that mutants blocked specifically at one of several morphological stages can be isolated, suggests that normal Bdellovibrio development must proceed in a sequential manner, that is, a developmental event(s) supplies the trigger for later events.

The observations on mutant 109D153 and the investigations of 109D nonspecific attachment to Bacillus subtilis may provide insights relevant both to the survivial of Bdellovibrio in nature and to previous studies on the early stage interaction of Bdellovibrio with its host (87,88).

The effect of temperature on the burst size of B. bacteriovorus 109D growing in E. coli B, as reported by Seidler and Starr (56), indicates that Bdellovibrio reproduction, as measured by burst size,

was greatest at the lowest incubation temperature studied, 25C. (Note that these experiments did not distinguish between differences in the degree of Bdellovibrio snake fragmentation and total progeny mass.) These findings and the ease of isolation of Bdellovibrio from soil and water at ambient temperatures (B. bacteriovorus 109D was originally isolated by Stolp from soil) would seem to indicate an ecological niche for Bdellovibrio at these temperatures.

Varon and Shilo (87) examined the relationship between temperature and the extent of attachment to E. coli of a B. bacteriovorus 109J population. The degree of population attachment at the end of a 20-min period was found to increase with temperature elevation increments of 5C and to demonstrate an optimum at 35C, with subsequent temperature elevation increments yielding decreased values. As reported by Varon and Shilo, a population of Bdellovibrio which demonstrates approximately 70% attachment at 35C demonstrates only slightly higher than 40% attachment at 20C, approximately 12% attachment at 15C, and even less significant attachment at lower temperatures. In another experiment in the same report, Varon and Shilo examined the effect of shift in the incubation temperature on the attachment of B. bacteriovorus 109J to E. coli. A shift in the incubation temperature of the parasite-host mixture from 20C (conditions which allow only part of the Bdellovibrio cells to attach) to 30C was found to result in a "new wave" of attachment up to the level achieved in mixtures incubated at 30C from the start.

These attachment experiments may reflect values resulting from the manifestation of two distinct attachment mechanisms. Peak

attachment values may represent an initial nonspecific interaction shifted towards completion by a specific, irreversible event. The attachment values obtained by Varon and Shilo for temperatures in the range of 15 to 20C seem to reflect ranges of attachment more compatable to our nonspecific attachment values (% A value of B. bacteriovorus 109D attachment to B. subtilis is 21%), which probably represent an equilibrium situation established between free swimming and attached Bdellovibrio forms. Lower attachment values at even lower temperatures may reflect a shift in this equilibrium by secondary changes in the physiology of the initial nonspecific parasite-host interaction event, most notably a change in Bdellovibrio motility.

Bdellovibrio attachment would thus appear to involve an initial nonspecific, reversible interaction which is followed by a temperature-stimulated, specific, irreversible event. Correlation of this view of attachment with the apparent preference of the Bdellovibrio burst size for less elevated temperatures would seem to lead to competition between the two processes. The net effects resolve at some optimal temperatire for Bdellobivrio parasitism of E. coli.

The degree to which the Bdellovibrio nonspecific, reversible attachment phenomenon may be operating at environmental temperatures has led us to speculations on the possible ecological-evolutionary advantages of such an attachment mechanism. This predation-divorced attachment to noncongenial hosts could function in effect as a reversible holdfast. The widespread distribution of holdfast organelles would seem to attest to their ecological-evolutionary potential and

speculation in print on the possible ecological role of the Caulobacter (48) and Pasteuria ramosa (65) holdfasts are brought to mind. The importance of such a device to so motile an organism as Bdellovibrio at first, however, appears moot. But observations have shown that Bdellovibrio lysates yield immobile, yet viable forms within an hour. To such an organism, the presence of a holdfast-like mechanism might be very beneficial, and the analogy to the Caulobacter situation becomes stronger.

This mechanism might thus form a basis for continued species endurance under conditions of individual hardship. As pointed out by Poindexter in a review on the genus Caulobacter (48), such attachment can be interpreted as a means for establishing a close association with a continuous source of nutrients excreted by other organisms. Thus, a microenvironment is established which is richer in nutrients than the surrounding milieu. Such an association would not be indefinitely beneficial but the sparing effect of glutamate, acetate, and amino acid mixtures upon Bdellovibrio endogenous respiration demonstrated recently by Rittenberg et al. (30) would appear to enhance the favorability of such an association.

Varon and Shilo (88), in their study on the attachment of B. bacteriovorus to cell wall mutants of Salmonella and E. coli, suggest that Bdellovibrio populations may be heterogeneous, consisting of (i) "cells capable of efficient attachment to both rough and smooth strains," and (ii) "cells capable of efficient attachment only to rough strains." They do point out, however, that the efficiency of

plating in their experiments was the same when two Bdellovibrio strains were plated on the seven cell wall variant host strains used. Our modified view of Bdellovibrio attachment, as representing a biphasic phenomenon comprised of reversible and irreversible stages, offers an alternative view for the proposed culture heterogeneity. In this case, variations in cell wall composition would be seen as influencing the rapidity of onset of the second, irreversible stage and thus affecting the rapidity and degree to which the attached, unattached equilibrium population is shifted towards completion.

The effects that the density-label 5-bromouracil might have when incorporated within an organism are best termed as variable. The ease of incorporation of 5BU in place of thymine probably reflects the fact that the van der Waals radius of the bromine atom (1.95 Å) differs only slightly from that of the methyl group (2.01Å) it replaces. However, the greater electronegativity of the bromine atom alters the electron distribution in the pyrimidine ring, and this may lead to a shift from the normal keto state to an enol state in which BU may pair with guanine instead of adenine. This keto-enol shift is thought to be responsible for the observed mutagenic effect of BU in DNA (36).

The incorporation of 5BU into the DNA of an organism has several effects not directly related to its mutagenic effect. These include a modification in the pattern of synthesis of one or multiple proteins, an alteration of differentiation of the organism, sensitization

to inactivation by ultraviolet light, X-rays or visible light, and an effect on viability which is very similar to the phenomenon of thymineless death (28). Incorporation of 5BU into DNA greatly increases the photosensitivity of that DNA. The major photochemical lesion in BU-DNA is a single-strand break. The initial event in the formation of the single-strand break is the photodissociation of the bromine atom to form a uracilyl radical. In the normal structure for DNA the bromine of BU is close to the deoxyribose sugar of the nucleotide in the same strand as the excited BU base, and on the 5' side. The radical formed upon photodissociation extracts a hydrogen atom from this sugar, forming the base uracil. The sugar, lacking a hydrogen, undergoes decomposition leading to cleavage of the phosphodiester bond (formation of a single-strand break), with the uracil on the 5' side (36).

The reason that DNA synthesis eventually stops in the presence of BU is not clear, but it has been suggested that the explanation may be the same as for loss of DNA-synthesizing capacity following thymine starvation (47). The phenomenon of thymineless death, first described by Barner and Cohen (4), remains poorly understood, although much information about it has been accumulated. The loss of viability during thymine deprivation was initially ascribed to unbalanced growth, as cytoplasmic syntheses continue in the absence of chromosonal replication. This concept was subsequently refined when it was shown that specifically RNA synthesis, but not protein synthesis, is a prerequisite for thymineless death (27). The forma-

tion of single-strand breaks and their repair has been closely linked to the induction of thymineless death (47).

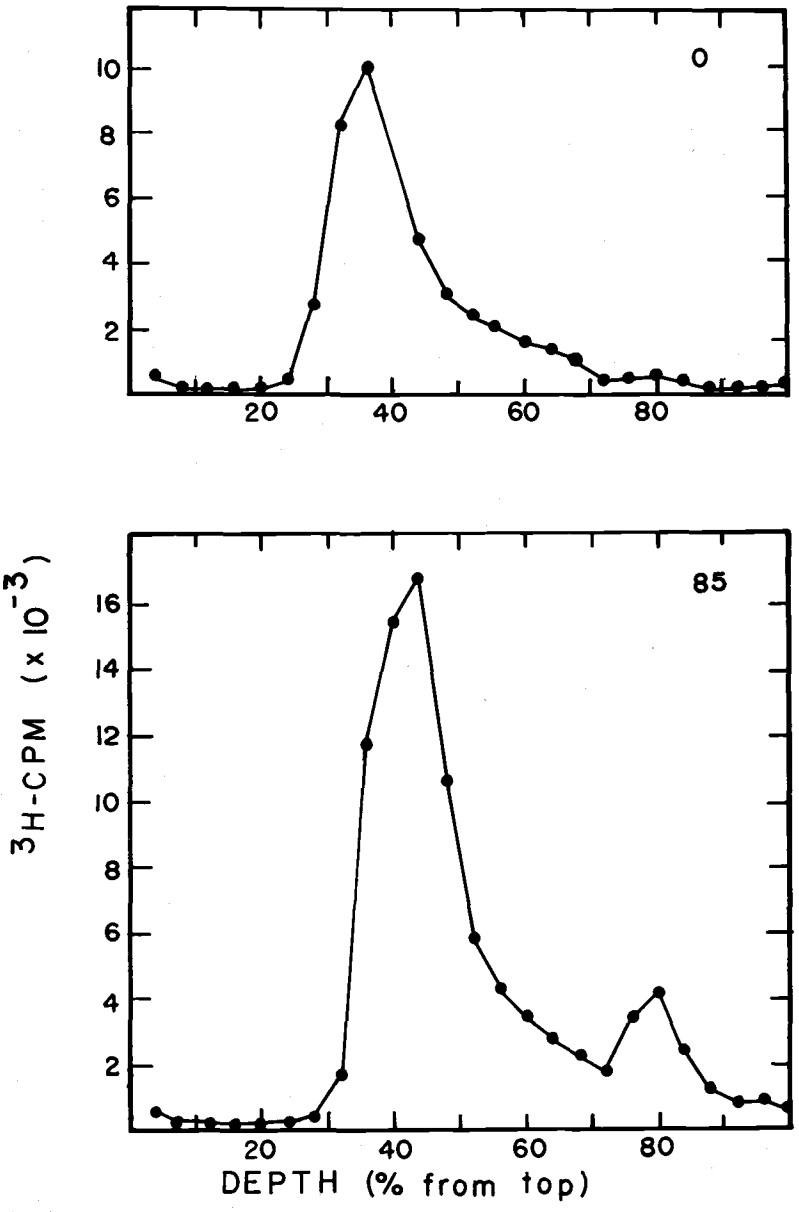
During the course of our density-label experiments with Bdellovibrio bacteriovorus, we found that although an initial light/heavy hybrid peak was formed by what would appear to be traditional semiconservative DNA replication, this peak rapidly reached a maximum. Although this peak was maintained upon continued incubation, evidence is presented which was interpreted as indicating the rapid onset of and increasing presence of repair-like replication (i.e., the incorporation of density-label in a random manner). Thus, it appears that although Bdellovibrio DNA synthesis is initiated normally under the conditions tested, Bdellovibrio DNA replication is acutely sensitive to the presence of 5BU. This sensitivity is expressed by the rapid onset of a thymineless death-like condition in which normal semiconservative DNA synthesis is replaced by extensive random, repair-like synthesis. This rapid onset of repair-like DNA synthesis invalidates the study of Bdellovibrio DNA replication via density-shift (5BU) experimentation.

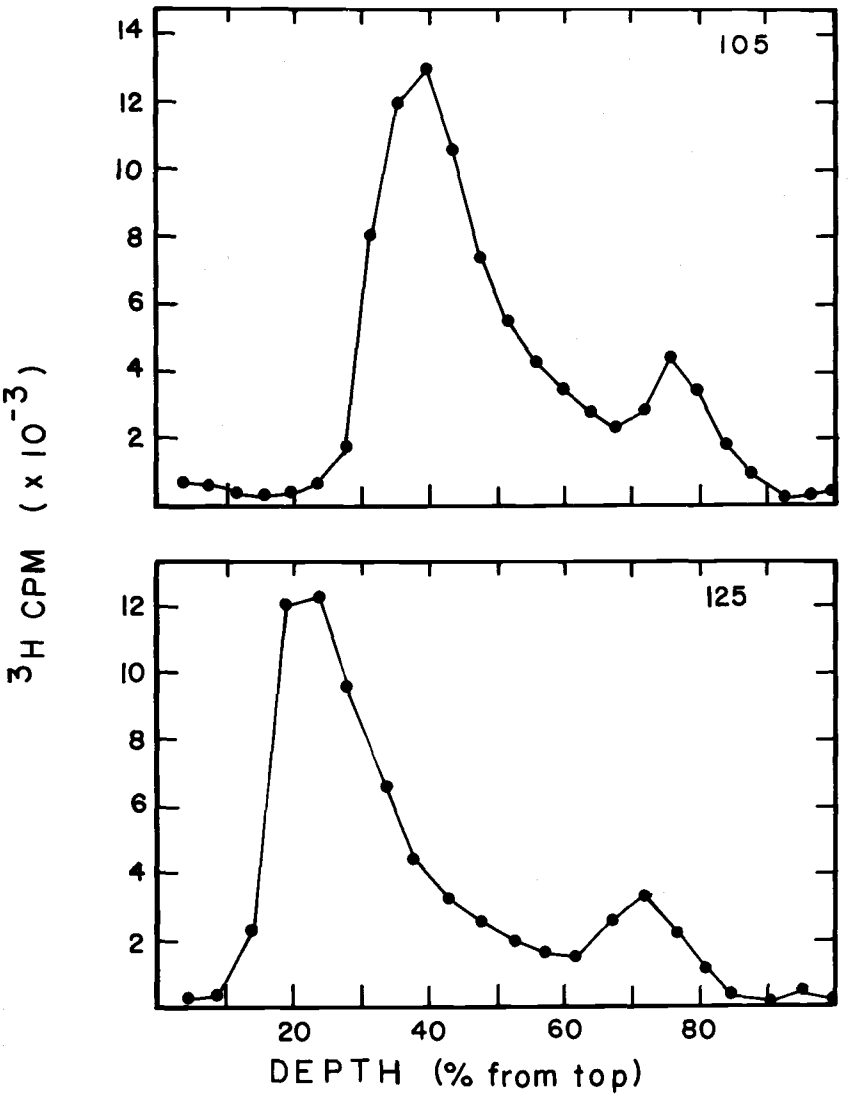
APPENDIX

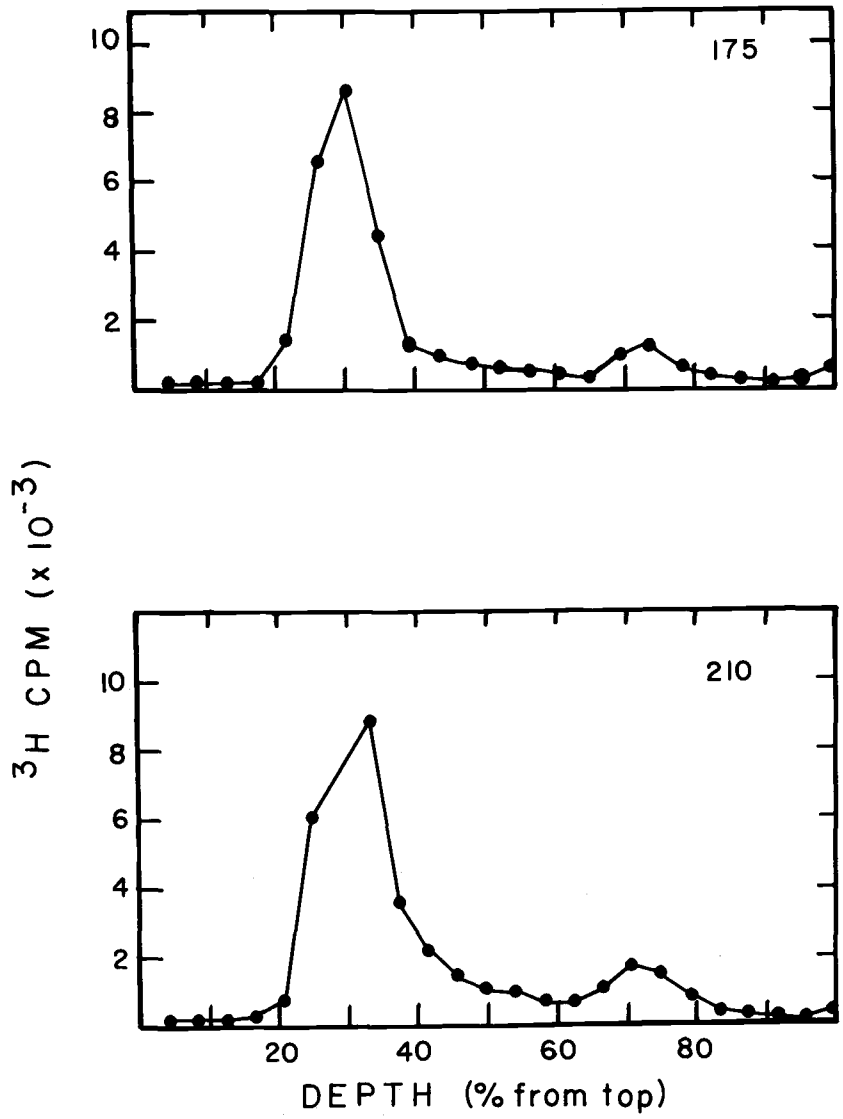
(Reference: page 62.)

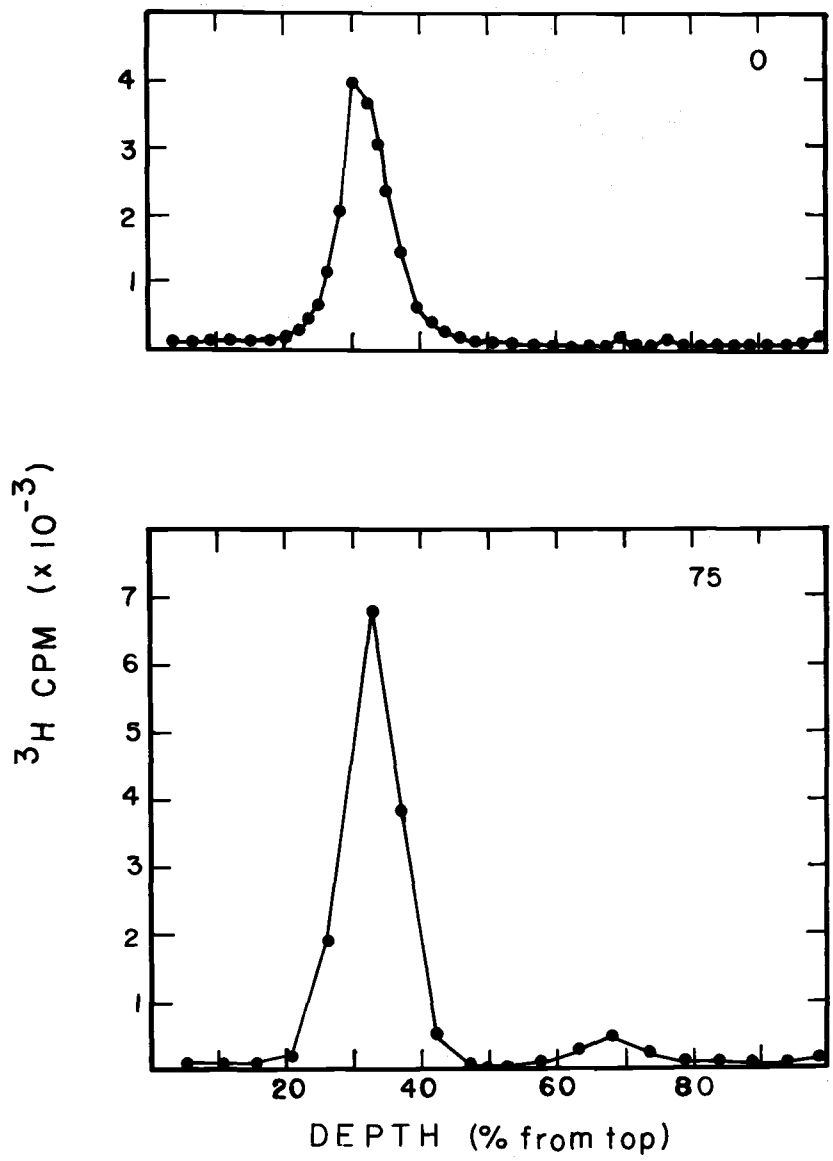
Figures 20-25. CsCl equilibrium density gradient analysis of the infection of 5BU substituted E. coli UC12 with Bdellovibrio bacteriovorus 109D.

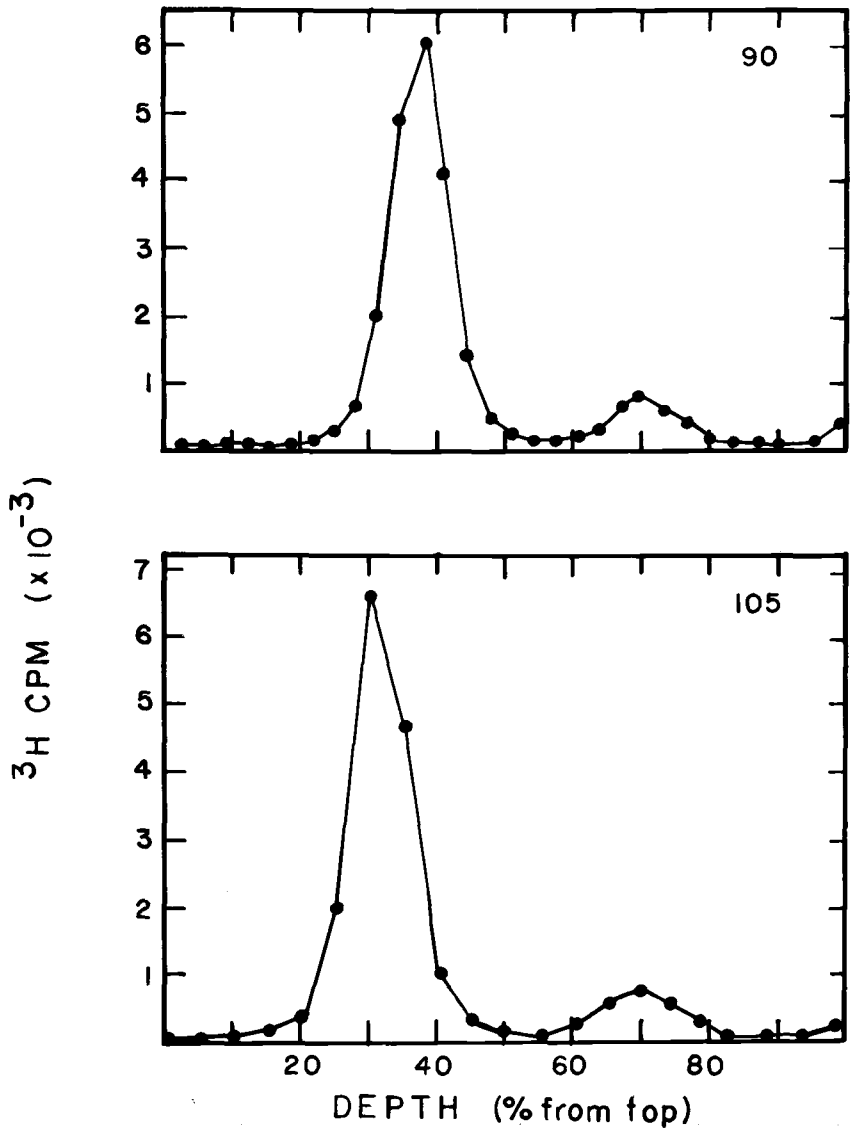
5BU substituted E. coli UC12 were infected under one-step growth conditions with isotopically labeled Bdellovibrio. At the times indicated following infection, samples were mixed with an equal volume of a chilled saline-EDTA-KCN killing solution, centrifuged and the cell pellets frozen at -20C. Subsequently the samples were thawed and the DNA extracted and submitted to CsCl equilibrium density gradient centrifugation. Fractions were collected from the top and the TCA precipitable counts determined.

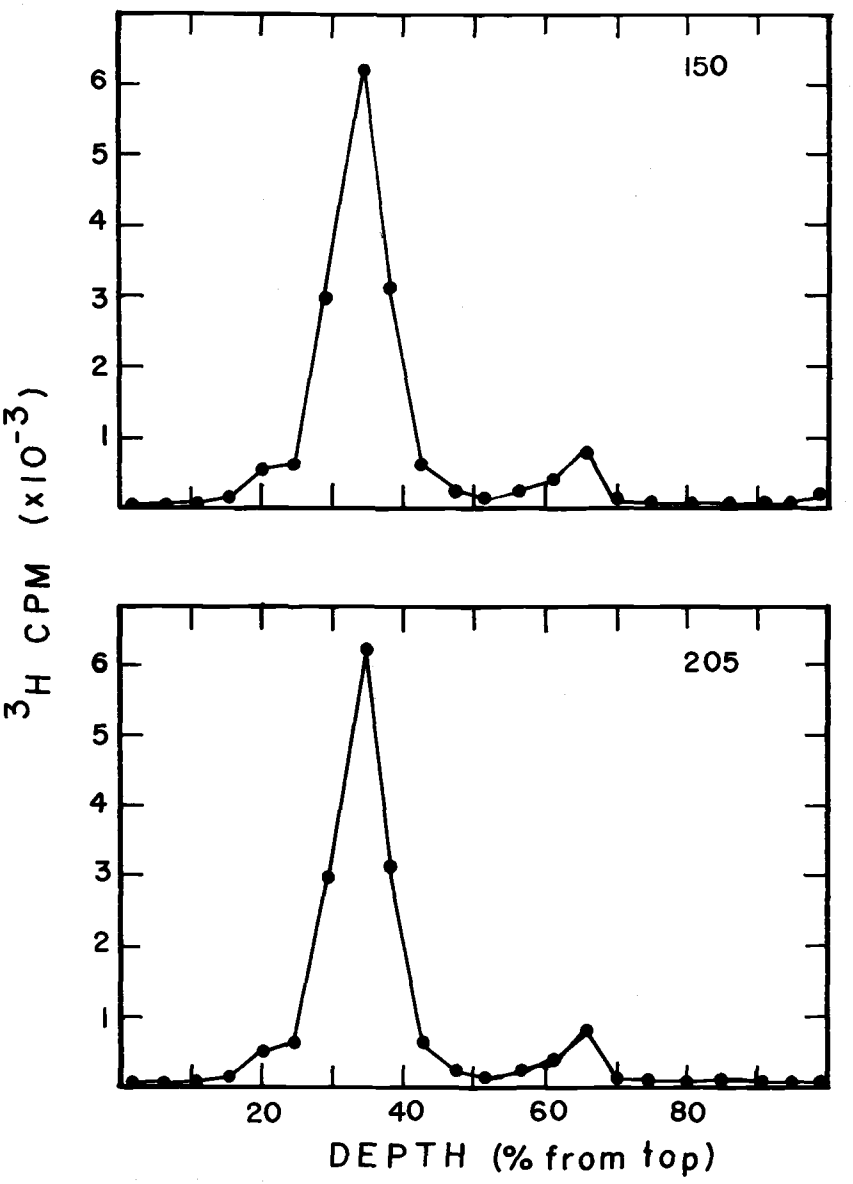

0
85
^{3}H-CPM (x 10^{-3})
DEPTH (% from top)
2
4
6
8
10
12
14
16
20
40
60
80


105
125
14
12
10
8
6
4
2
20
40
60
80
^{3}H CPM (x 10^{-3})
DEPTH (% from top)


175
210
10
8
6
4
2
20
40
60
80
^{3}H CPM (x 10^{-3})
DEPTH (% from top)


0
75
^{3}H CPM (x 10^{-3})
DEPTH (% from top)
1
2
3
4
5
6
7
20
40
60
80


90
105
^{3}H CPM (x10^{-3})
DEPTH (% from top)
20
40
60
80


150
205
6
5
4
3
2
1
20
40
60
80
^{3}H CPM (x10^{-3})
DEPTH (% from top)

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