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Title: DETERMINATION OF DIFFERENTIAL GENE ACTION IN

BDELLOVIBRIO BACTERIOVORUS USING

RNA-DNA HYBRIDIZATION TECHNIQUES

Abstract approved:

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In an attempt to study the times in the life-cycle of
Bdellovibrio bacteriovorus at which various genes are
transcribed, the patterns of messenger ribonucleic acid
(m-RNA) and ribosomal ribonucleic acid (r-RNA) syntheses
were examined using two different types of hybridization
systems. The hybridization system using membrane bound
deoxyribonucleic acid (DNA) and saturating amounts of RNA,
provides the fraction of DNA which is transcribing RNA at
various time periods in the organisms life-cycle. The
hybridization system using DNA in solution in excess of the
RNA, provides the fractions of the the total RNA being
transcribed at the various time periods in the organisms
life cycle. Using pulse labeled RNA from host-dependent
Bdellovibrio bacteriovorus 109(Davis), (HD 109D), incubated
in a casamino acids medium, the fraction of DNA
transcribing RNA at that time period in the life-cycle was
found to be 4.3% of the total DNA, or 8.6% of the potential

gene weight of the cell. Competition with unlabeled r-RNA purified from host independent Bdellovibrio bacteriovorus 109(Davis), (HI 109D), in late logarithmic growth showed that 48% of the transcribed DNA, or 2% of the total DNA, was producing 65% of the total labeled RNA as r-RNA. This amount of DNA was calculated to code for 13 ribosomal cistrons. Total RNA extracted from HD 109D grown through a complete developmental cycle in Spirillum serpens cell sonicate, and RNA extracted from HD 109D grown only to the mid-point in its life-cycle in S. serpens cell sonicate was also used as cold competitor. Both competed for 92% of the sites on the DNA available to the labeled HD 109D RNA and made up 81% of the total labeled RNA. Total RNA extracted from HI 109D in logarithmic growth also competed successfully for the DNA sites available to 81% of the labeled RNA. This indicates that 92% of the DNA transcribed during the labeling period is also transcribed during the time periods from which the competitor RNA was extracted. As r-RNA was demonstrated to comprise 48% of the transcribed DNA cistrons, this leaves 44% of the transcribed DNA producing 16% of the m-RNA during all the time periods tested, and 8% (that fraction which retained the labeled RNA after competition) producing 19% of the m-RNA unique to the labeling period.

Determination of Differential Gene Action in <u>Bdellovibrio</u>
<u>bacteriovorus</u> Using RNA-DNA Hybridization Techniques

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INTRODUCTION

Bdellovibrio bacteriovorus has the unique ability to penetrate a variety of gram negative bacteria and then utilize the contents of the host cell for its own growth and division. This utilization is very efficient, in part because of the precise regulation by the Bdellovibrio of the degradation and resynthesis of the host cell material into Bdellovibrio cell products (4,7,8,16,26). It has been inferred from the study of these processes that the vibrio possesses a complex series of interacting inducible genes. In the present study, the sequence and nature of messenger ribonucleic acid (m-RNA) and ribosomal ribonucleic acid (r-RNA) species produced during various phases of the life cycle of Bdellovibrio were examined using two different types of hybridization systems. One type, introduced by Whitfield et. al. (18), uses a small amount of labeled RNA in solution with a large amount of denatured deoxyribonucleic acid (DNA). Under the proper conditions of temperature and buffer, DNA-DNA renaturation and DNA-RNA hybridization occur concurrently. If the RNA involved in

the hybridization reaction does not significantly reduce the concentration of the denatured DNA, then the two reactions (hybridization and renaturation) will occur independently of each other (18). In this type of system, since all the RNA present has the potential to form hybrid complexes (12,18), great sensitivity is gained in competition experiments in detecting the proportions of different RNA species present. The actual amount of RNA hybridizing to the DNA will depend on the relative rates of hybridization and renaturation which are a function of salt, temperature, and nucleic acid concentration (12,18).

The other type of competition/hybridization system was introduced by Gillespie and Spiegelman (6) and involves the binding of denatured DNA to nitrocellulose filters. Enough labeled RNA is added to fill all the sites on the DNA which were being transcribed during the time period in which the RNA was labeled, thus showing the approximate fraction of DNA involved in transcription during the labeling period. Kennell used RNA-DNA hybridization techniques to titrate the gene sites on the Escherichia coli chromosome (12). He showed that at RNA/DNA ratios of just >1/4, genes for m-RNA just begin to become saturated. Stable RNA saturated its gene sites at an RNA/DNA ratio of approximately 1/160.

Yasumoto and Doi (37) used the methods of Gillespie and Spiegelman (6) to determine the nature of the differential gene action occuring during the various stages of the

sporulation of Bacillus subtilis. Using pulse labeled RNA obtained from various stages during the sporulation process of the organism and competing with unlabeled heterologous RNA, they demonstrated a sequential increase in the amount of sporulation-specific transcription from the DNA and a reduced rate of transcription of genes active during the logarithmic growth phase of the bacterium. They were hampered in the interpretation of their results however, by the fact that they didn't know what proportion of the total RNA was being contributed by the different genes being transcribed. They were therefore unable to determine the relative importance of the different genes transcribed.

Bhagwat and Mahadevan (1) ran similar experiments investigating differential gene action in Neurospora crassa with similar results. That is, they were able to demonstrate a difference in the type and number of actively transcribed genes during the time periods tested, but could not determine the relative importance of the gene product to the organism in terms of the relative amounts present.

The present study uses both hybridization/competition techniques to determine the patterns of DNA transcription and the importance to Bdellovibrio of the resulting RNA in terms of the relative amounts produced.

LITERATURE REVIEW

Bdellovibrio bacteriovorus is unique among bacteria in having the ability to grow in the periplasmic region of any of a number of diverse gram negative bacteria (28,31,33). Their predatious interaction with sensitive bacteria, beginning with a forceful contact of the rapidly motile bdellovibrio with a congenial host, initiates a complex series of events. The small vibrio penetrates its host and lodges between the cell wall and cytoplasmic membrane. Cell growth begins, with concomitant digestion of the host cell intracellular menstrum. Cell growth may continue for two to three hours or longer. It is very efficient, with just over half of the cell carbon of the substrate organism being converted into bdellovibrio cell material (21,26,36). The elongated spiral or C-shaped organism undergoes sequential fragmentation, and progeny parasites are released.

Seidler and Starr (26) have shown, using the host-dependent strain of Bdellovibrio bacteriovorus 109 Davis (HD 109D), that divalent cations such as calcium and magnesium are required for the attachment of the Bdellovibrio to the host cell wall, and for stabilization of the host cell membrane once the Bdellovibrio has breached the cell wall. The pH optimum of the organism is broad, lying between 7.0

and 8.5. The temperature optimum lies between 30-35° C. No growth occured at 42° C.

There are three recognized species in the genus Bdellovibrio. Bdellovibrio bacteriovorus is represented by strains 100, 109D, 6-5-S and others with a guanine-cytosine (GC) base content of the deoxyribonucleic acid (DNA) ranging from 49.5 - 51.5%, and a genome about 60% the size of Escherichia coli (E. coli). The DNA-DNA homology among isolates in this group is more than 90%. There is no detectable homology between B. bacteriovorus and the other two species of Bdellovibrio.

Strain A 3-12 is designated B. starrii and is the only known member of this species. The GC content is 43.5%, the genome is about 75% the size of E. coli, and it has only 16% homology with B. stolpii.

B. stolpii is represented by strain Uki-2. It has a 41.8% GC composition and a genome size about 67% of that of E. coli. It also is the only known member of this species.

Much is being learned about the metabolism of Bdellovibrio. Research in this area is complicated however, because bdellovibrios require host cells and cannot be grown on conventional defined or complex media. The nature of this host dependence is not clear; however some information has been gained by the isolation of host independent mutants. Host-independent (HI) mutants of host-dependent (HD) bdellovibrios were discovered by the isolation of

colonies that appeared on host-free media inoculated with HD bdellovibrios. The HI mutants are apparently physiologically identical to the HD parent strain, except for the loss of motility by some strains and the non-requirement of a host (27).

Because of the frequency of HI mutants in an HD population (10^{-6} to 10^{-7}), a point mutation is believed to be responsible for the HI derivitives of HD bdellovibrios. The exact nature of this mutation is still in doubt, but studies of the nutritional requirements of the HI and HD bdellovibrios have revealed some interesting information. Early studies on an HI mutant of Bdellovibrio strain 321 showed it to be gram negative, aerobic, proteolytic, (hydrolyzing both casein and gelatin), and unable to grow on a variety of carbon sources except complex media containing protein (7,34).

Using an HI derivitive of A 3-12, Shilo and Bruff (29) found that thymidine could partially replace the requirement of the organism for yeast extract. They also showed that a minimum of 10^4 organisms per plate was necessary to obtain colony formation on nutrient agar, and that the addition of autoclaved culture supernatent fluids to the medium, to a final concentration of 1%, eliminated this inoculum dependence.

Using HD 109D, Ishiguro (11) followed up this study by giving closer attention to the requirement of spent culture

supernatent for growth of HI bdellovibrios, since this requirement would indicate the necessity of the presence of some growth factor (or factors) to initiate growth of HI derivitives of Bdellovibrio. He found that the growth initiation factor (GIF) appeared to be produced by Bdellovibrio or released upon lysis of host cells. Cell free extracts were prepared from a number of different bacteria, both host and non-host for Bdellovibrio, by sonication of the cultures, high speed centrifugation to remove cell debris, and dialysis of the supernatent against Tris(hydroxymethyl) aminomethanehydrochloride (Tris) buffer. The GIF was present in all extracts, was stable to heating and freezing, and was associated with both the soluble and particulate portions of the extract. The requirement for the GIF was limited to small inocula of stationary phase HI cells. Furthermore, this requirement disappeared with continued laboratory maintainence of the cultures.

Reiner and Shilo (20), in a slightly different approach, used microbial extracts to attempt to culture HD Bdellovibrios outside the host organism. Using both host and non-host cells for Bdellovibrio, they prepared cell free extracts similarly to Ishiguro (11); that is, with sonic treatment of the cells, high speed centrifugation, and dialysis of the supernatent. Using phase contrast microscopy and thymidine incorporation experiments, they estimated that approximately 5% of the HD 109J population

began synthesis and growth. This occured only in the cell free extracts. As found by previous investigators, no DNA synthesis or growth occured in other complex media. Reiner and Shilo found the factor in the microbial extract which initiated HD 109J growth and DNA systhesis to be non-dializable, heat stable, and RNAase, DNAase and pronase resistant.

In a follow-up study, Horowitz et.al. (10) used E. coli B extracts, prepared as described above except for the addition of divalent cations, to study the growth of several HD Bdellovibrio strains. In this case, about 50% of the bdellovibrios underwent changes typical of an HD type in a host bacterium. There was an early loss of flagella, an elongation into filaments, and multiple fission into flagellated progeny. The morphological changes occuring in the cells were correlated with DNA synthesis as measured by labeled thymidine incorporation. The host extract was required continuously during the growth cycle, and filament length and time of division depended on its concentration. Removal of the Bdellovibrios from the extract caused cessation of thymidine incorporation and eventual fragmentation of the cells into flagellated progeny. If the extract was treated with either ribonuclease (RNAase) or pronase before the addition of the HD 109J, there was neither growth nor thymidine incorporation, contrary to previous results (20).

The observations reported suggest that HD Bdellovibrio depends on the presence of some host factor for the initiation of DNA systhesis, and its depletion triggers cell division. Whether or not this factor is the same as that investigated by Ishiguro in relation to the induction of HI Bdellovibrio growth is not known. Because of procedural differences, the studies cannot be compared. The exact nature of the action of the GIF in the host cell extract is also in doubt. It may be that the host is supplying to the HD Bdellovibrio a factor which it cannot produce, and that the mutation to the HI form involves the acquisition of the ability to manufacture this factor. Alternatively, it has been suggested that the host factor interacts with a repressor in the HD Bdellovibrio, thus activating the necessary pathways needed for DNA synthesis and for balanced growth. The mutation to the HI form may involve loss of this repressor (10).

Furthur investigation into the nature of the use of host material by Bdellovibrio has shown it to be remarkably organized and efficient. The host cell supplies all the carbon and energy material required by the Bdellovibrio for growth and multiplication. This has been shown by growth of HD Bdellovibrio in buffer solutions containing only host cells (26). Bdellovibrio is also independent of any of the metabolic systems of its host. This has been demonstrated by growing HD Bdellovibrios on heat killed or UV killed host

cells or in cell free host extracts as previously mentioned (3,10,20). Several studies have shown that amino acids, especially glutamate and alanine, are the primary carbon and energy source for Bdellovibrio metabolism (7,25,29). The comparative studies by Seidler, Mandel, and Baptist (25) confirm the one made by Simpson and Robinson (30). Both demonstrate a functional Krebs cycle as well as alanine and glutamine dehydrogenases. Assays for glucose-6-phospho-gluconate dehydrogenases gave negative results.

Engelking and Seidler (5) demonstrated the incorporation of labeled amino acids into cold trichloroacetic acid (TCA) precipitable material, but not into hot TCA soluble material, indicating no detectable level of de novo biosynthesis of purines and pyrimidines. Ninety five percent of the material from both labeled E. coli hydrolyzed DNA and RNA did become soluble in hot 5% TCA. Isotope tracer studies suggest that thymidine and uracil are the breakdown products of the nucleic acids which are incorporated by the Bdellovibrio because they are readily taken up and thymine and uridine are not.

Hespell et. al. (7) made a detailed study of the destruction of host cell RNA and its resynthesis as Bdellovibrio nucleic acid. Using (5-3H) uracil labeled HD 109J and (2-14C) uracil labeled E. coli, they showed that the E. coli ribosomal RNA (r-RNA) was almost completely degraded within the first 90 minutes of a 210 to 240 minute

growth cycle. Synthesis of Bdellovibrio r-RNA was not detected until after 90 minutes using this prelabeled high molecular weight host nucleic acid as the nutrient source. The r-RNA present in the bdellovibrios at the time of infection of the E. coli remained fairly constant throughout the development cycle. About 50% of the total radioactivity in the E. coli was incorporated into the bdellovibrios; 66% of this found its way into the HD 109J RNA and the other 33% was found in the DNA. The total radioactivity in the Bdellovibrio DNA was approximately three times that initially present in the E. coli DNA, indicating that the E. coli RNA served as precursor for the Bdellovibrio RNA and for some of its DNA as well. The rest of the label was released into the surrounding medium as free nucleic acid bases. Mixtures of nucleic acid bases or ribonucleosides had little effect on the amount of radioactivity taken up. In contrast, the addition of the four ribo or deoxyribonucleoside monophosphates caused a 25% to 35% decrease in incorporated radioactivity. They concluded that the RNA of the substrate cell was degraded to the form of nucleoside monophosphates and served as the exclusive precursor for the Bdellovibrio RNA and for some of its DNA as well.

Similar types of studies by various people have shown essentially the same pattern for all the metabolic activities of Bdellovibrio. Matin and Rittenberg (16), and

Rittenberg and Langley (22) showed a similarly regulated breakdown of substrate cell DNA and its subsequent resynthesis from the resulting deoxyribonucleoside monophosphates. Kuenen and Rittenberg (13) demonstrated that the fatty acids of Bdellovibrio are derived almost exclusively, and with little or no alteration, from the lipids of the substrate organism.

The above listed properties of Bdellovibrio suggest that its intraperiplasmic development should occur with a minimum of energy expenditure. Rittenberg and Hespell (21) investigated the energy efficiency of HD 109J by calculating the grams (dry weight) of cell material formed per mole of ATP, (Y-ATP). They found the Y-ATP for Bdellovibrio grown in single-cycle experiments to be 18.5, and the Y-ATP for Bdellovibrio grown in multi-cycle experiments to be 25.9. This compares to an average of 10.5 for most other bacteria. However, because Bdellovibrio has a wide variety of pre-formed monomers available from the substrate organism, and because it utilizes nucleoside monophosphates (7,22) and complete fatty acids (13), its energy requirements are reduced, and the theoretical Y-ATP increases proportionately. Even allowing for these energy sparing effects, the ratio of the observed Y-ATP to the theoretical Y-ATP they calculate to be .52-.73 for Bdellovibrio, as compared to the .36 ratio found for most other bacteria. Rittenberg and Hespell explain this high Y-ATP of the

Bdellovibrio through consideration of energetic coupling during growth. Failure to make effective use of ATP produced by catabolism results in energetic uncoupling. The ability of the Bdellovibrio to regulate the degradation of the substrate cell, and its control of the types, times of appearance, and rates of formation of biosynthetic units could result in a very high degree of coupling between anabolic and catabolic processes, which would explain its unusual energy efficiency.

In conclusion, wild type Bdellovibrio requires host cells for a factor to initiate growth and division (either by supplying a product the organism can't synthesize or by combining with a repressor) and a supply of carbon and energy sources, and possibly vitamins. The Bdellovibrio strictly regulates the destruction and resynthesis of host cell components such that 50% or more of the host cell carbon becomes part of the Bdellovibrio. The efficiency of macromolecular syntheses by Bdellovibrio is 1.5 to 2 times greater than that of other bacteria.

MATERIALS AND METHODS

Organisms and Media

A host-dependent and a host-independent strain of
Bdellovibrio bacteriovorus 109(Davis), (HD 109D and HI 109D
respectively), were used in these studies (26).   The host
organism used to support the growth of HD 109D was Spirillum
serpens MW5.

S. serpens and HI 109D were grown separately in a broth
medium (designated PYE), which consisted of 1.0%
bacto-peptone (Difco), 0.3% yeast extract (Difco), and 0.01M
Tris(hydroxy- methyl)aminomethanehydrochloride buffer at pH
7.5 (Tris, Calbiochem). This is the medium used by Siedler
and Starr (27). Cultures were grown by inoculating 5 mls of
a 12 hour culture into 50 mls of PYE.

HD 109D was propagated in a ten-fold dilution of PYE
(PYE/10), to which had been added autoclaved solutions of
calcium chloride dihydrate and magnesium sulfate to final
concentrations of 0.002M and 0.003M respectively (cations)
(26). For routine maintenance and cultivation, 5 mls of a
12 hour culture of S. serpens was added to 30 mls of PYE/10
along with the cations and 0.3 to 0.5 mls of a 12 hour
culture of HD 109D. For the larger cultures needed for the

extraction of RNA from HD 109D, 250 mls of a 12 hour culture of S. serpens was centrifuged in a Beckman J-21 centrifuge at approximately 6000 x g for 10 minutes. The resulting pellet was resuspended in 0.001M Tris buffer to a volume of about 50 mls, and was then added to 250 mls of PYE/10 with cations and 30 mls of a 12 hour lysate of HD 109D.

Preparation of S. serpens Cell Sonicate
for the Growth of HD 109D

The host-free cell extract prepared for the growth of HD 109D varies significantly from that of Reiner and Shilo (20) and from Horowitz et. al. (10). High speed centrifugation of the sonicate for removal of all cell debris was not used; neither was the sonicate dialized against a buffer. Sterilization was accomplished by heat treatment rather than by filtration, and no nutritional supplements were added as in the other studies (10,20).

The cell sonicate of S. serpens was prepared by centrifuging a 24 hour culture of the organism at approximately 6000 x g for 10 minutes, washing it in 0.001M Tris buffer, centrifuging it again as indicated above and then resuspending it in 0.001M Tris buffer to a concentration giving an optical density (O.D.) reading of twenty (after extrapolation from suitable dilutions), at 600nm. The O.D. was determined with a Bausch and Lomb

Spectronic 20. Chilled aliquots of 30 mls were sonicated for a total of 27 minutes using two minute pulses of sonic treatment followed by one minute intervals to allow cooling of cell material. The sonicate was then centrifuged at 200 x g for 15 minutes to sediment unbroken cells. The pellet was discarded and the supernatent was stored at -20° C. Prior to use, the protein concentration was determined by the Lowry method (14) and then the sonicate was diluted in 0.001M Tris buffer, pH 7.5, to a final concentration of 1.7 to 1.8 mg of protein per ml. This was heated at 60 C° for one hour and incubated at 31° C for 6 to 8 hours between three repeated 60° C heatings to sterilize it.

To enumerate HD 109D by counting plaques, a double agar overlay method was used (10). The base agar consisted of PYE/10 with cations (0.002M $CaCl_2$ ($2H_2$O),0.003M $MgSO_4$), and agar at a concentration of 0.8% (wt/vol). The overlay agar was composed of PYE/10 with cations, and agar at a concentration of 0.6% (wt/vol). Each plate was covered with 3 mls of overlay agar to which had been added 1 ml of a twelve hour culture of S. serpens and 0.1 ml of HD 109D.

Preparation of HD 109D for Inoculation into
0.1% Casamino Acids or S. serpens Sonicate

One step growth conditions for HD 109D were produced by modifications of the procedures of Seidler and Starr (27)

and Varon and Shilo (35). A 12 hour lysate of HD 109D was centrifuged for 15 minutes at 200 x g at 5° C. The supernatent was then sequentially filtered through 5um and 1.2um nitrocellulose membrane filters (Millipore) to remove any remaining S. serpens and cell debris. The filtrate was then centrifuged at 25,500 x g at 5° C for 20 minutes. The Bdellovibrio pellet was washed and resuspended in 0.001M Tris plus cations to give an absorptance reading of 1.5 at 600nm. This corresponds to a cell density of 1 to 2 x 10^{10} plaque forming units (PFU) per ml. A 10% (v/v) inoculum was placed into 0.1% casamino acids or a 15% (v/v) inoculum was placed into S. serpens cell sonicate. These cultures were then incubated on a New Brunswick gyrotory shaker at 210 rpm and at 30° C. The casamino acids culture was incubated for 35 minutes and the cell sonicate culture was incubated for either 2.5 or 5 hours. HD 109D does not increase in PFU in the casamino acids medium. In S. serpens cell sonicate, the bdellovibrios complete one growth cycle in about 5 hours as determined by plaque assay and microscopic observations. The burst size was about 7 to 10 x 10^{9} Bdellovibrio per ml when incubated in the sonicate.

Preparation of Labeled

HD 109D RNA

The host-dependent bdellovibrios were incubated in the

casamino acids medium for 25 minutes. Tritiated (5-3H) uracil (New England Nuclear), was added to a final concentration of 5.0 uCi/ml and the culture incubated an additional 10 minutes. At this point, sodium azide was added to a final concentration of 0.001M and the HD 109D was immediately centrifuged at 25,500 x g for 20 minutes at 5°C. The pellet was washed in one-third its original volume in a solution of 0.15M sodium chloride and 0.1M ethylenediaminotetra-acetic acid, pH 8, (saline-EDTA). The cells were then resuspended to approximately one-tenth their original volume in saline-EDTA and lysed with sodium dodecyl sulfate (SDS), which was added to a final concentration of 2.5%. The RNA extraction was continued, following the procedure of Moore and McCarthy (19). Non-radioactive homologous competitor was obtained by the same procedure.

Extraction of Ribosomal RNA

A culture of HI 109D was grown to late log phase by transferring a 50 ml inoculum of the cells to one liter of PYE and incubating at 31° C for two hours on the New Brunswick shaker at 210 rpm. The ribosomes were isolated from the cells by using a modification of the method used by Ron, Kohler and Davis (23). The cells were pelleted by centrifugation at 10,000 x g for 10 minutes at 5° C. The pellet was resuspended to 1% of the original volume in iced

0.01M Tris, pH 7.75, with 0.001M magnesium acetate buffer. Lysozyme was added to a concentration of 1.0 mg/ml and the cells were frozen by swirling the flask in acetone and dry ice. The cells were slowly thawed in cool water. They were then subjected to two additional freeze-thaw cycles. DNAase (Sigma, electrophoratically pure) was added to a final concentration of 10 ug/ml. The cells were kept chilled, and lysed by adding 0.3 mls of a 10% solution of sodium deoxycolate for each 10 mls of cell suspension. The lysate was then centrifuged at 30,000 x g for 20 minutes at 5° C to remove cell debris. The ribosomes and their subunits were isolated by centrifuging the supernatent at 105,000 x g for 4 hours (2). The pellet was resuspended in saline-EDTA and the ribosomal ribonucleic acid (r-RNA) purified using the standard techniques of Moore and McCarthy (19).

Preparation of Competitor RNA from
HD 109D Grown in Host Cell Sonicate

HD 109D was inoculated into S. serpens cell sonicate as previously described. Pilot experiments on 30 ml volumes showed that the HD 109D would complete a growth cycle in 5 hours. Growth of the cultures was terminated with sodium azide either after the end of the growth cycle at 5 hours or midway, at 2.5 hours. The one step growth

was complete. When it was to be interrupted at the halfway point, a 10 ml aliquot was transferred to a sterile 125 ml flask before the sodium azide was added and this was examined microscopically to confirm that the growth cycle was completed after 5 hours. After the addition of sodium azide, the cultures were immediately centrifuged at 25,500 x g at 5° C for 20 minutes. The pellet was resuspended in one-third its original volume in saline-EDTA, washed, resuspended in the same buffer, and the RNA extracted as described above.

Preparation of HI 109D
for RNA Extraction

RNA from HI 109D grown to the late logarithmic phase was used as one of the specimens of competitor RNA. To obtain this RNA, a 5% inoculum of the Bdellovibrio was made into PYE and incubated at 31° C for 24 ± 2 hours. The cells were then centrifuged at 10,000 x g for 10 minutes at 5° C. The pelleted cells were washed in saline-EDTA at approximately one-fourth their original volume, then resuspended in the same buffer to one-tenth of the original volume and the RNA extracted as described.

Tests for RNA Purity

Each RNA sample was routinely assayed for freedom from protein contamination in a U.V. spectrophotometer (Beckman Instrument Inc.). The absorptance readings at 230nm, 260nm, 280nm, and 320nm, were recorded. If the ratios of the absorptances of the 260nm/280nm readings and the 260nm/230nm readings were 1.9 $\pm$ 0.05 and 2.3 $\pm$ 0.1 respectively, the sample was considered acceptable (7) and dialyzed six hours against 50 to 100 volumes of hybridization buffer. The buffer was changed and dialysis was continued an additional 10 to 12 hours. The buffer was composed of 0.3M NaCl and 0.03M tri-sodium citrate (2xSSC), pH 7.0, and 30% dimethylsulfoxide (DMSO, Mallinckrodtt).

The labeled RNA was tested for freedom from labeled DNA by several means before the hybridization experiments. It was found to be totally hydrolyzed, (less than 3% of the cold 5% trichloroacetic acid precipitable counts remained), after 40 minutes in 5% trichloroacetic acid (TCA) at 85°C, after one hour in 100 ug/ml heat treated pancreatic ribonuclease (RNAase) in 2 X SSC at 37°C, or after 18 hours in 0.1N NaOH at 37°C.

Extraction and Purification of DNA

Cultures of either HI 109D or S. serpens were grown for

24 hours in PYE broth, then harvested by centrifugation at 5° C. The HI 109D was centrifuged at 10,000 x g for 10 minutes and the S. serpens was centrifuged at 6,000 x g for 10 minutes. The pellets were washed, then resuspended in saline-EDTA. Cells were stored frozen, suspended in saline-EDTA until needed.

Extraction and purification of the deoxyribonucleic acid (DNA) followed a modification of Marmur's procedure (15). Neutralized saline-EDTA equilibrated phenol was used for primary deproteinizations and a solution of chloroform plus 4% isoamyl alcohol (Sevag) was used for subsequent ones.

Each DNA sample was routinely assayed for the degree of purification in the Beckman U.V. spectrophotometer. The absorptance readings at 230nm, 260nm, 280nm and 320nm were recorded. If the 260nm/320nm ratio was 2.3 ± 0.1 and the 260nm/280nm ratio was 1.9 ± 0.02, the DNA was considered acceptable and stored at 4°C over a drop of chloroform.

Determination of the Melting Point
of HD 109D DNA and S. serpens DNA

DNA was sheared in a French Press (Aminco), at 15,000 psi yielding fragments with a double stranded molecular weight of approximately 1.0×10^6 daltons (15). The sheared DNA was dialyzed in hybridization buffer (2xSSC with 30%

DMSO) at a DNA to buffer ratio of 1/50 or less. The buffer was changed after six hours and the DNA dialyzed an additional 9 to 12 hours. Samples were placed in quartz cuvettes and stoppered. The hyperchromic shift was recorded at 260 nm as the temperature was raised at the rate of about 2° C every 5 minutes. The T_m of both HI 109D and S. serpens DNA was found to be 73^oC.

Immobilization of DNA
on Membrane Filters

All materials were precooled to 4^oC. The HI 109D DNA was loaded onto membrane filters (Schleicher and Schuell Co., B-6; 15 cm diameter) by the procedure of Gillespie and Spiegelman (6). Three milligrams of native DNA were dissolved in 100 mls of 0.1xSSC. The DNA was denatured with 10 mls of 1N NaOH for five minutes, then diluted with 500 mls of 1.0M NaH_2PO_4. The denatured DNA was loaded onto the filters which had been washed with 200 mls of 2xSSC and marked with a pencil to indicate the confines of the area to contain the DNA. The DNA was allowed to gravity load overnight at 4^oC. Any remaining solution was filtered using a slight vacuum of 5 psi. The filters were then washed with 100 mls of 6xSSC, drained on paper towels to remove excess moisture, then dried for 16 hours at 60° C to 80° C in a vacuum desiccator. After drying, a paper punch (GEM) was

used to punch smaller filters, 6.5 mm in diameter. These were placed in vials and stored under vacuum in a refrigerated desiccator.

The amount of DNA on each filter was determined from the absorptance obtained at 260 nm on the Beckman spectrophotometer after some of the filters had been placed in 0.5 mls of 0.5N $HClO_4$ and heated at $70^{\circ}C$ for 20 minutes (16). The average amount of DNA on each filter (before hybridization) was determined to be 15 ug on the basis that 1 OD at 260 nm is obtained at a concentration of 50 ug of DNA per ml (24). The variation in the concentration of DNA on the filters was $\pm$ 8.5%.

Preparation of Hybridization Buffer

The buffer used for membrane filter hybridization and for hybridization in solution was 2xSSC, plus 30% DMSO by volume. Purified RNA and sheared DNA were dialized against this buffer and stored in the freezer until needed. As the salts tended to precipitate when mixed directly with the DMSO, the 10xSSC stock solution (pH 7.0), was diluted before the DMSO was added.

Preparation of Dialysis Tubing

The tubing, (Van Waters & Rogers, regenerated

cellulose), was cut into convenient lengths and placed in a stainless steel beaker with a 10% solution of sodium carbonate. A weighted flask was placed on top to keep the tubing in direct contact with the solution and it was heated in a steamer for 2 hours. The dialysis tubing was then rinsed thoroughly in cold tap water, then in running distilled water for an hour. It was stored at room temperature in 70% ethanol.

RNA-DNA Hybridization Procedure
Using Membrane Filter Bound DNA

Membrane filters with approximately 15 ug of HI 109D DNA bound to them were placed in one-half dram vials containing a total volume of 0.5 mls of hybridization buffer with 200 ug of 3H RNA from HD 109D, and 0-4000 ug of cold competitor RNA from either HD or HI 109D. The vials were tightly capped and incubated in a waterbath at $48^{o}C$, (T_m-25° C), for 16 to 18 hours. The filters were then removed and washed in three consecutive washes of the hybridization buffer heated to T_m-25° to remove the nonspecifically binding RNA. The filters were then immersed in 0.5 mls of 2xSSC containing pancreatic RNAase at a concentration of 20 ug/ml for one hour at $37^{o}C$. The filters were then rewashed in three consecutive rinses of 2xSSC to remove the DMSO. This was also at a temperature of T_m-25°. The filters were dried

overnight at $45^{\circ}C$ and counted. The amount of RNA binding to the filters was determined by its specific activity and the counts retained on the filters.

To determine the amount of DNA left on the filters after the hybridization procedure, a separate set of eleven filters with DNA bound to them were put into 0.5 mls of hybridization buffer and incubated in the waterbath along with the competition experiment. The DNA remaining on the filters after the hybridization procedure was eluted from them (and from the filters from the competition experiment which had had no unlabeled RNA added to them). This was accomplished by heating the filters in 0.5 mls of 0.5N $HClO_4$ at $70^{\circ}C$ for 20 minutes (16). The filters which had labeled, but not unlabeled RNA hybridized to them, were first soaked for 10 minutes in each of three changes of toluene, after the hybridized RNA had been counted, to remove the omnifluor. The filters were then dried overnight at $45^{\circ}C$ to evaporate the toluene before they were heated in the $HClO_4$. The amount of DNA eluted from the filters was determined from the absorptance at 260 nm using the relationship of 1 OD_{260} =50 ug/ml of DNA (24). A Beckman spectrophotometer was used for this purpose. For the filters with the labeled RNA bound to the DNA, the amount of RNA present had been determined previously and was therefore subtracted from the absorptance reading at 260 nm according to the relationship 1 OD_{260}=44 ug/ml of

RNA (2) before the amount of DNA present was calculated.
The average amount of DNA eluted from the filters by the
$HClO_4$ treatment, (the amount retained by the filters during
the hybridization procedure), was determined to be 9.2 $\pm$ 5
ug.

RNA-DNA Hybridization in Solution

200 ug of sheared DNA, 0.3 ug of 3H HD RNA, and 0 to
1500 ug of unlabeled HD or HI 109D cold competitor RNA were
placed in small (13 mm x 100 mm) test tubes with a silicon
(Clay Adams, Siliclad) coating. All nucleotides had been
previously dialyzed against the hybridization buffer and
this buffer was added where necessary to bring the total
volume in each tube to 0.5 mls. The tubes were tightly
capped, their contents thoroughly mixed, then placed in a
steamer and heated for 7 to 8 minutes to denature the DNA
(18). Immediately after removal from the steamer, the
tubes were put into an ice-water bath to prevent premature
renaturation of the nucleic acids (18) before they were
placed in a water bath at 53°C (T_m-20°) for 12 to 14 hours.
To remove the non-hybridized RNA, heat treated pancreatic
Ribonuclease-A was added to a concentration of 100 ug/ml
and incubated at 37°C for 1.5 hours. The tubes were then
chilled on ice and an equal volume of cold 10% TCA was
added to each. Thirty minutes after the cold TCA

precipitation, the contents of the tubes were filtered through 1.2 um nitrocellulose filters (Millipore). Each tube and filter was subsequently washed with 10 mls of 5% TCA and 10 mls of cold 70% ethanol. The filters were then placed in scintillation vials, dried overnight at $37^{\circ}C$, and counted. The amount of RNA which hybridized was determined from the counts and the specific activity of the RNA.

The specific activity of the RNA was determined in the presence of 200 ug of DNA to take into consideration the quenching effect of the DNA. The maximum amount of hybridization was determined by following the previously described hybridization procedure but not adding any unlabeled competitor RNA. Background counts were subtracted from all other counts and were determined by running the 0.3 ug of labeled HD 109D RNA through the described hybridization procedure alone, adding the DNA after the RNAase treatment.

Determination of the Quenching
of Counts by Competition RNA

To determine if the tremendous amount of RNA needed for competition was affecting the results through the quenching of counts, (which would be interpreted as increased competition), the following test was performed: 200 ug of HI 109D DNA and 0.3 ug of HD 109D 3H-RNA were

combined to a total volume of 0.6 mls in hybridization buffer then TCA precipitated, filtered and counted as described previously. The values obtained by this procedure give the total possible counts.

To determine if RNA which is not RNAase treated will cause quenching, 1000 ug of cold competitor RNA derived from the bdellovibrios incubated in the casamino acids media was added to the standard amount of HI 109D DNA and labeled RNA, TCA precipitated, filtered and counted as previously described.

Finally, to find out if RNAase treatment is sufficient to eliminate any quenching which might occur, 1000 ug of the unlabeled RNA was added to 200 ug of the HI 109D DNA. After incubation of this mixture with 50 ug of heat treated RNAase at 37°C for 1 hr, the sample was TCA precipitated and 0.3 ug of labeled RNA was then added. The sample was then filtered and counted as previously described.

The results (Table 1), show that insufficient RNAase treatment will indeed cause a quenching of the counts. The RNAase treatment used is sufficient to minimize the problem and the use of the same amount of competitor for all competition experiments should negate its effects since all samples will be equally affected.

TABLE 1

Effect of RNA on the Quenching of Counts

	CPM Sample 1	CPM Sample 2
Total counts per minute (no competition RNA and no RNAase added)	836.7	822.8
Counts per minute when 1000 ug cold RNA is added but not RNAase treated	448.2	436.3
Counts per minute when 1000 ug cold RNA is added and RNAase treated	748.9	764.3

Measuring Radioactivity

The radioactivity of TCA precipitates and of the RNA-DNA hybridization filters was measured by liquid scintillation counting using a fluor composed of one gallon of toluene into which 15.16 g of Omnifluor (New England Nuclear, 98% PPO and 2% Bis-MSB), was dissolved. Counting was done in a Nuclear Chicago Mark I scintillation counter. All samples were counted for 10 minutes in 5 mls of fluor. Counting efficiency and quenching were determined by the channel ratios method.

RESULTS

Determination of the Optimum

Time for Pulse Labeling

A continuous labeling experiment was conducted to determine the characteristics of (5-3H) Uracil uptake by host dependent Bdellovibrio bacteriovorus 109(Davis) (HD 109D). This was done to determine the optimum time at which to add the labeled uracil to the casamino acids in order to obtain the largest amount of label uptake in the shortest time period. The HD 109D was prepared and added to the casamino acids medium as described in Methods and Materials, along with (5-3H) Uracil at a final concentration of 0.4 uCi/ml. The culture was incubated on a gyrotory shaker at 30°C for 60 minutes. Samples of 0.1 ml were taken at periodic intervals and pipetted into 1.0 mls of cold 5% trichloroacetic acid (TCA). After 30 minutes, the samples were filtered through 1.2 um membrane filters (Millipore). The filters were washed with cold 70% ethanol, dried, and counted. A graph of the results (Fig. 1) shows that a 10 minute pulse after 20 minutes of incubation should provide sufficient labeling of the ribonucleic acid (RNA) synthesized during this early portion of the life-cycle.

FIGURE 1

(5-3H) Uracil Uptake of HD 109D in 0.1%
Casamino Acids Media at 0.4 uC/ml of (5-3H)
Uracil
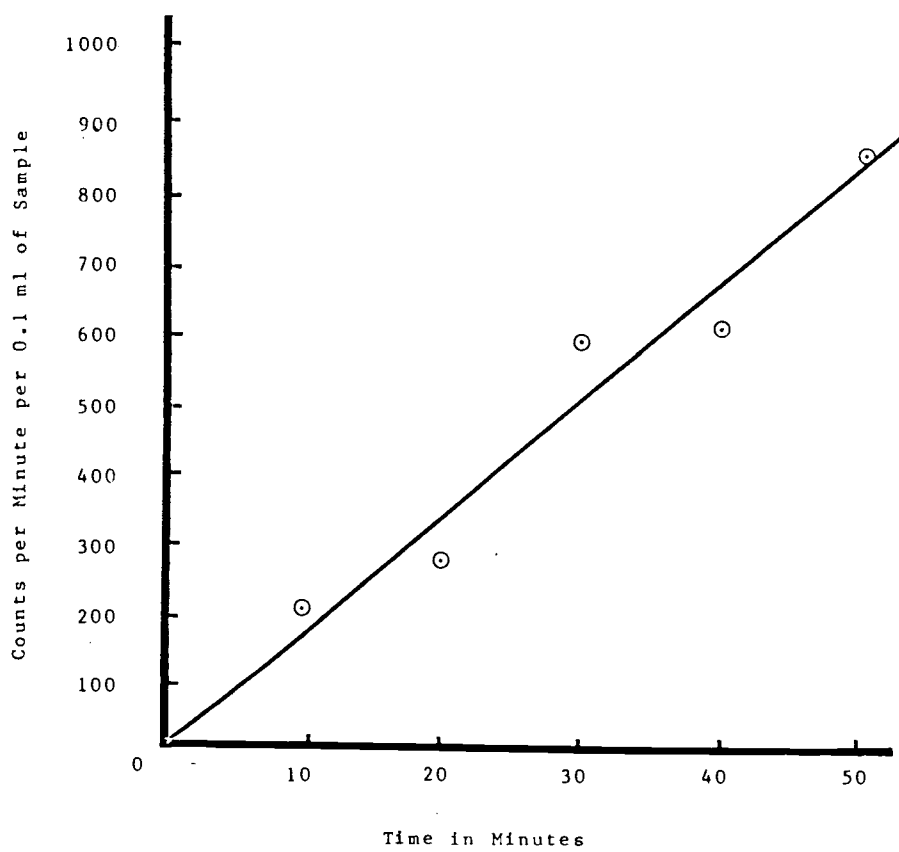
Determination of HD 109D Growth

Kinetics in Host Cell Sonicate

The growth kinetics of HD 109D in host cell sonicate were followed both microscopically and by plate counts of plaque forming units (PFU) in order to determine the kinetics and relative syncrony of growth. Microscopic determination of the end of the growth cycle was found to be in excellent agreement with the end point found by graphing PFU's against time (Fig. 2). The total time for an HD 109D culture to fragment from its large non-motile form to its smaller motile form under these conditions is 20 to 30 minutes. Growth of the Bd. bacteriovorus was therefore deemed to be sufficiently syncronous to allow the use of this method of culture to produce a population of cells with an anticipated similar RNA content at any point in time.

Determination of the Optimal RNA/DNA

Ratio for Hybridization in Solution

Varying amounts of labeled RNA (0.2, 0.3, 0.5 ug) from HD 109D incubated in the casamino acids medium were each hybridized with 100, 300, and 500 ug of deoxyribonucleic acid (DNA) from host independent Bdellovibrio bacteriovorus This range of values was chosen because other investigators

FIGURE 2

Growth Kinetics of HD 109D in Host Cell Sonicate

Plaque Forming Units X 10^9

Time in Hours
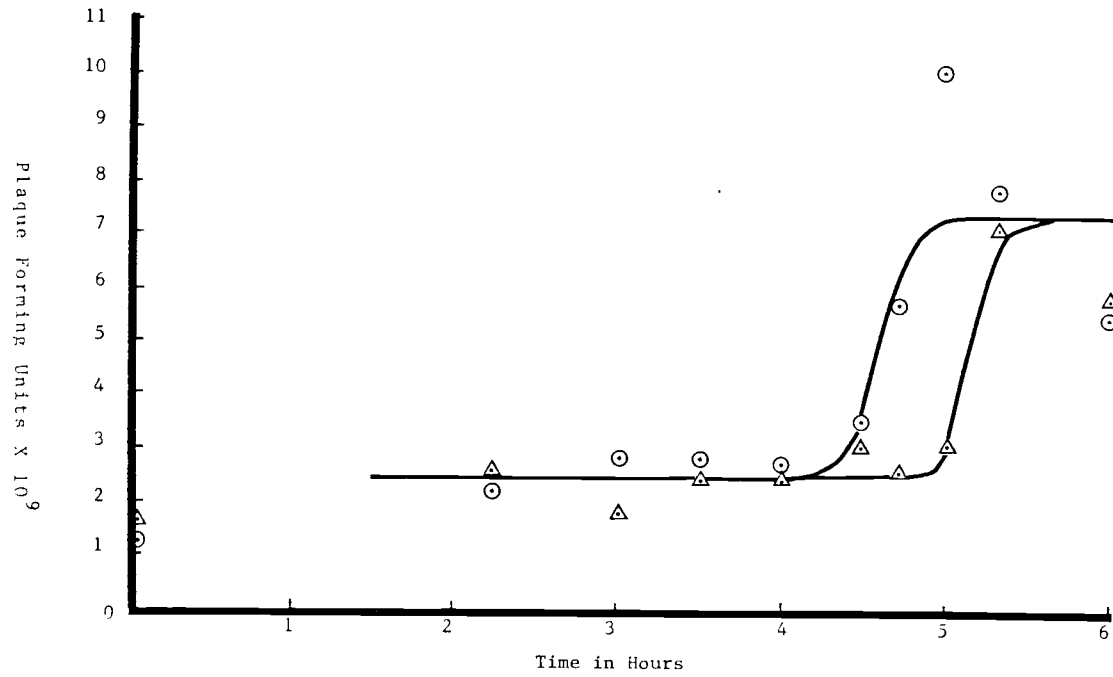
⊙ Flask number 1

△ Flask number 2

have found that the best RNA/DNA ratio for hybridization in solution is on the order of 1/1000 (12,18). The results are tabulated in Table 2. Total possible counts per minute were determined by passage of duplicate samples through the routine hybridization procedure described in Materials and Methods except for the elimination of the ribonuclease (RNAase) step. Hybrid counts were determined by hybridizing the various RNA/DNA mixtures and then adding RNAase to remove the non-specifically hybridized and non-hybridized RNA as described in Materials and Methods. Background counts were determined as outlined in Materials and Methods.

The data show two interesting trends. One is that increasing amounts of DNA cause an increased quenching of counts. The other is that the increase in the ratio of DNA to RNA has only a slight affect on the amount of RNA which will hybridize. In order to obtain a more detailed picture of the hybridization characteristics, a hybridization experiment was carried out keeping the labeled RNA input constant at 0.3 ug, and altering the amount of input DNA in small increments through the range of 1/666 to 1/1333. A similar experiment, but using Escherichia coli nucleic acids (12), resulted in a 30% increase in the total amount of RNA which hybridized. The data from this test (Table 3) does not show any indication that the increasing amounts of DNA resulted in any corresponding increase in the amount of

TABLE 2

Determination of the Optimal RNA/DNA
Ratio for Hybridization in Solution

ug RNA Added	ug DNA Added	Background Counts	Total Counts	Hybrid Counts	% of Total RNA Hybridizing(1)
0.2	100	29.0	456.8	127.8	23
0.2	300	31.1	241.1	84.1	25
0.2	500	33.8	135.5	60.7	26
0.3	100	30.9	682.3	149.1	18
0.3	300	37.7	369.0	110.4	22
0.3	500	-	161.4	72.4	30 (2)
0.5	100	41.2	1128.7	281.1	22
0.5	300	43.8	705.5	174.7	20
0.5	500	45.6	346.1	122.9	26

1. The percent hybridization achieved was calculated in
    the following manner:
    (Hybrid Counts)-(Background Counts)   % Total RNA
    (Total Counts)-(Background Counts)   Hybridizing

2. The background count subtracted from the total and
    hybrid counts was taken to be the average of the other
    background counts obtained when using 0.3 ug of
    tritiated RNA.

3. Each sample was run in duplicate. Each count per
    minute shown here is the average of the two obtained.

TABLE 3

Verification of the Optimal RNA/DNA
Ratio for Hybridization in Solution

ug DNA Added	Background Counts	Total Counts	Hybrid Counts	% of Total RNA Hybridizing (1)
200	34.3	777.3	335.9	41
230	34.4	783.1	333.4	40
265	34.3	715.6	300.2	39
300	32.2	656.0	263.5	37
330	35.4	632.2	280.5	41
365	35.2	614.5	273.5	41
400	34.3	622.4	250.5	37

1. The percent hybridization achieved was calculated
   in the following manner:

   $$\frac{(Hybrid\ Counts)-(Background\ Counts)}{(Total\ Counts)-(Background\ Counts)} \qquad \begin{array}{c} \%\ of\ Total\ RNA \\ Hybridizing \end{array}$$

   Duplicate samples were used in this experiment. Each
count per minute shown is the average of the two values
obtained. The amount of tritiated RNA added remained
constant at 0.3 ug.

RNA which hybridized. A maximum hybridization of approximately 40% of the input RNA has apparently been reached with 200 ug of DNA and 0.3 ug of labeled RNA.

To ensure that this figure represented a true hybridization maximum, and was not an artifact of the nucleic acid concentration or the length of time of hybridization, a brief study of the hybridization kinetics was carried out (Fig. 3). Labeled HD 109D RNA (0.3 ug) was combined with 200 ug of HI 109D DNA in a total volume of 0.5 mls or 1.5 mls of hybridization buffer. Hybridization was carried out with duplicate samples of each volume being pulled at successive time intervals and subjected to RNAase treatment. The expected hybridization maximum is 70 to 80% of the input RNA (12,18). The results show that the maximum hybridization achieved in this system is 35 to 40% of the input RNA, which is in agreement with the previous experiment. This maximum is reached after six hours of incubation, but longer time periods do not appear to affect the results.

Competition Curves

Competition curves in the liquid hybridization system were run using each of the cold competitor RNA's to locate the point at which maximum competition occurred. The curves (Fig. 4) were generated using 200 ug of HI 109D DNA,

FIGURE 3

Percentage of Labeled HD 109D Total RNA

Hybridizing to HI 109D DNA vs. Time
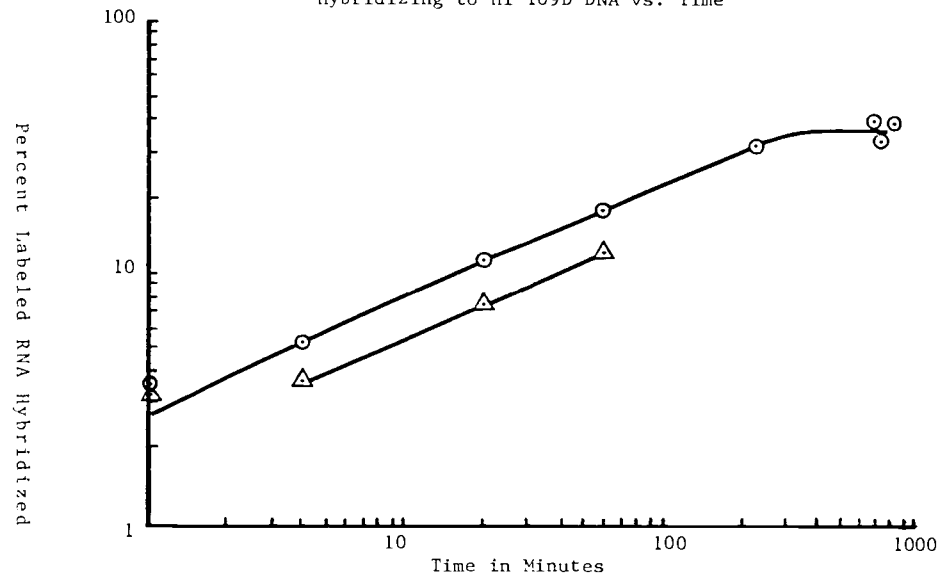
⊙ Hybridization carried out with 200ug HI 109D DNA and 0.3ug tritiated
   HD 109D total RNA in 0.5 mls hybridization buffer.

△ Hybridization carried out with 200ug HI 109D DNA and 0.3ug tritiated
   HD 109D total RNA in 1.5 mls hybridization buffer.

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FIGURE 4

Competition Experiment Using 0.3 ug Tritiated Total RNA
from HD 109D Pulse Labeled 10 Minutes in Casamino Acids
Media and 200 ug HI 109D DNA.
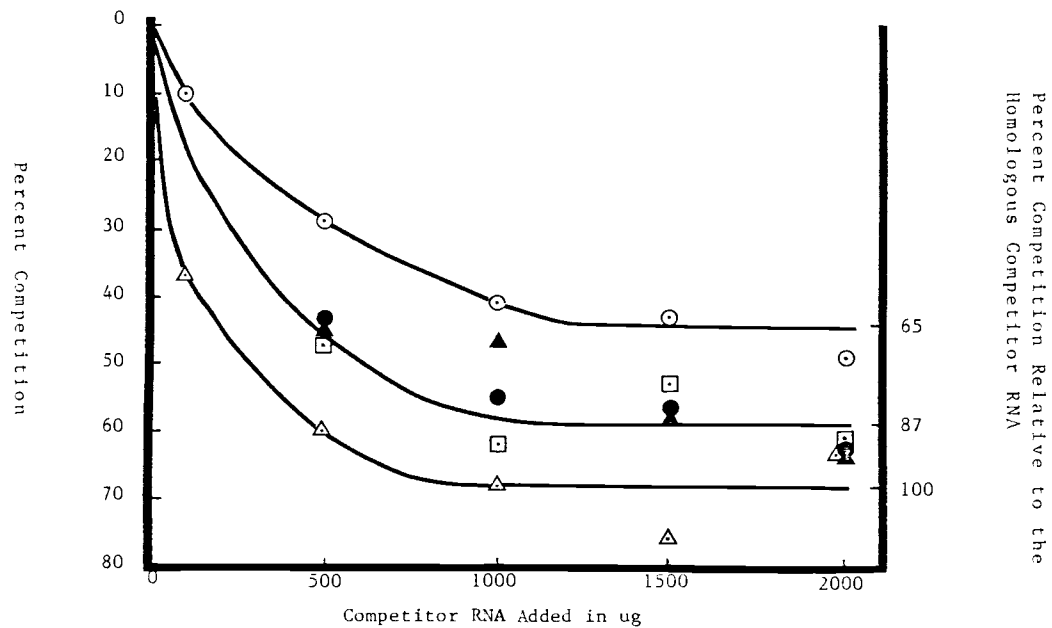
Cold Competitor RNA's Used:

⊙ HI 109D r-RNA
▲ HI 109D total RNA
● HD 109D total RNA from a culture grown for a full life-cycle
  in host cell sonicate.
⊡ HD 109D total RNA from a culture grown to mid-cycle in
  host cell sonicate.
△ HD 109D RNA homologous to the labeled RNA

40

0.3 ug tritiated HD 109D RNA, and 0 to 2000 ug cold competitor RNA from HD 109D or HI 109D.

The results show that 1) 1500 ug of competitor RNA is the minimum amount which can be used and still obtain maximum competition, 2) r-RNA appears to comprise about 65% of the labeled RNA extracted from the HD 109D incubated in the casamino acids medium, and 3) there is a difference of about 13% between the amount of competition produced by homologous competitor RNA and that produced by the competitor RNA derived from HI 109D and from HD 109D grown in host cell sonicate. The latter difference should represent the percentage of m-RNA produced by the vibrios in the casamino acids medium which is unique to the non-homologous competitor RNA species. Since total RNA was used as competitor (unless otherwise stated), the r-RNA should not be responsible for any significant part of this difference.

Statistical Examination of
the Competition Results

Because of the scattering of values obtained for the experiments presented in Fig. 4, a number of additional competition experiments were run using only 1500 ug of competitor. This would permit a statistical test to determine the most probable true values of the competition

produced by the different competitor RNA's. Each sample was treated separately and not averaged with any others. The data from using r-RNA and homologous RNA as cold competitor was first plotted into bar graphs (Figs. 5 & 6) to determine the manner in which the points were distributed. These graphs indicate a normal distribution of the values around the mean, so a two-tailed t-test at the 95% confidence interval was used to determine the most probable range of values for the means (Tables 4 & 5).

The ranges of the means, for competition achieved with homologous competitor RNA and r-RNA, are distinctly different from each other and from those of the other three RNA's. The r-RNA competed for 45% of the DNA sites avalible to the labeled RNA (65% of the relative amount of competition obtained using homologous RNA), which is nearly identical to the value indicated by the competition curves of Fig. 4. The competition values obtained from the non-homologous total cold competitor RNA derived from HD 109D and from HI 109D have very similar means, and the ranges overlap to the point of being identical. These data indicate that (100-81) 19% (rather than 13% as first indicated in Fig. 4) of the RNA produced by the HD 109D in the casamino acids medium is m-RNA different from the m-RNA found at any of the other time periods sampled. This does not say however, whether the unique RNA species produced under these three conditions are identical to each other.

FIGURE 5

Bar Graph Showing the Distribution of Competition
Values Obtained with Unlabeled Homologous RNA
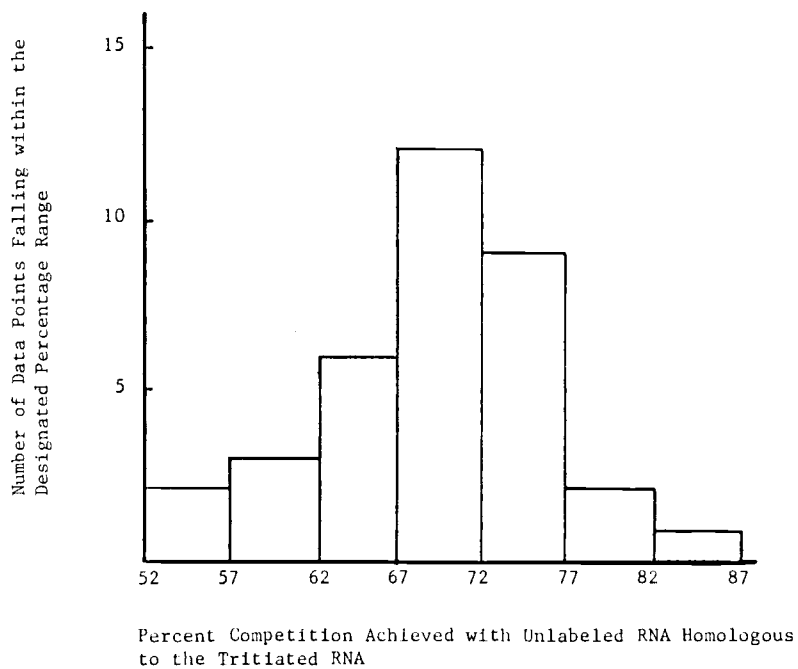

Percent Competition Achieved with Unlabeled RNA Homologous
to the Tritiated RNA

FIGURE 6

Bar Graph Showing the Distribution of Competition
Values Obtained with Unlabeled HI 109D r-RNA

Percent Competition Achieved with Unlabeled r-RNA
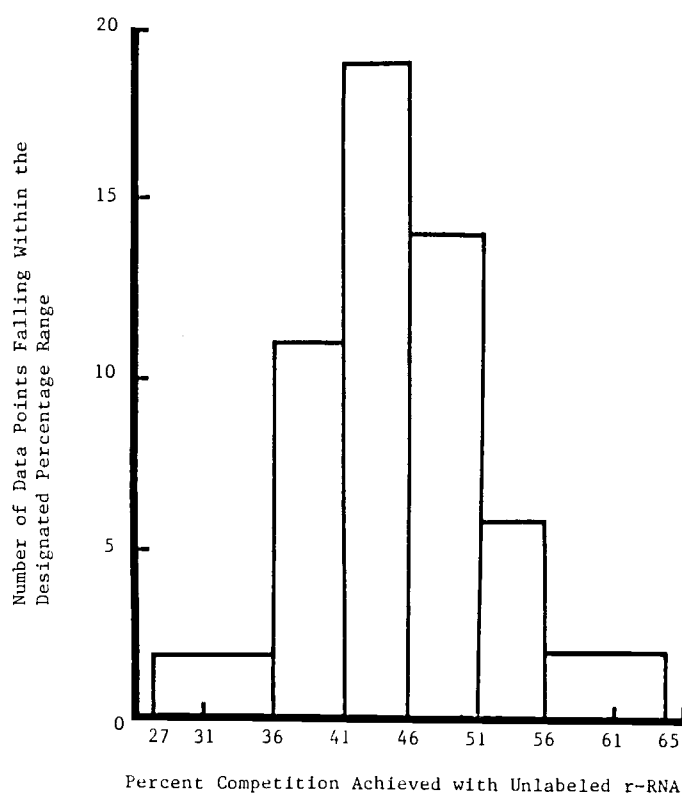
TABLE 4

Data table illustrating the percent competition achieved in the liquid hybridization system using 0.3 ug of tritiated RNA, 200 ug of HI 109D DNA, and 1500 ug of competitor RNA from:

HD 109D incubated in casamino acids media
(homologous cold competitor)

55.22	56.76	58.37	58.44	59.22	63.12	63.49	64.68
64.76	65.27	66.88	67.39	67.90	68.40	68.47	68.60
69.03	69.47	69.67	69.81	69.84	71.60	71.81	72.50
72.79	73.06	73.31	73.48	75.09	74.40	75.79	76.31
76.76	77.99	80.16	82.94				

HD 109D incubated 5 hours in host cell sonicate

48.63	49.70	50.19	50.53	51.35	53.02	53.09	53.48
53.86	54.46	57.03	57.85	58.41	58.92	59.80	68.49

HD 109D incubated 2.5 hours in host cell sonicate

51.84	52.75	52.83	54.31	55.14	55.47	56.89	58.44
62.12	62.94	67.47					

TABLE 4 (continued)

HI 109D grown to late log phase

40.30	43.19	44.49	45.66	47.16	47.36	47.77	48.95
52.60	52.68	56.24	58.12	60.46	61.61	63.14	64.05
69.76	69.98	72.58	73.40	74.33			

HI 109D ribosomal RNA

31.31	35.27	36.78	37.42	37.83	38.14	38.50	38.69
39.55	39.82	39.92	40.02	40.49	41.24	41.30	41.52
41.75	41.87	42.24	42.50	43.06	43.62	43.70	44.09
44.78	45.01	45.36	45.40	45.50	45.55	45.55	45.87
46.06	46.14	46.19	46.42	46.55	46.55	46.59	47.27
47.21	47.81	47.95	48.05	48.72	49.72	51.16	51.27
51.89	52.79	52.99	53.88	56.07	64.27		

TABLE 5

Mean Competition Value Ranges at the 95% C.I.

	RNA from HD 109D held in casamino acids (Homologous RNA)	RNA from HD 109D grown for 2.5 hrs in host cell sonicate	RNA from HD 109D grown for 5 hrs in host cell sonicate	RNA from HI 109D	r-RNA from HI 109D
Number of data points (n)	36	11	16	21	54
Degrees of freedom (df)	35	10	15	20	53
Mean % value of competition $(\overline{X})$	69.27	57.29	54.93	56.85	44.80
Standard deviation (s)	6.56	4.97	5.04	10.95	5.67
$(s/\sqrt{n})$	1.09	1.50	1.26	2.39	0.772
(t_{05})	2.030	2.228	2.131	2.086	2.0056
$(s/\sqrt{n}) \times (t_{05})$	2.21	3.34	2.69	4.99	1.55
Range of $\overline{X}$ at the 95% C.I.	67.1-71.5	54.0-60.6	52.2-57.6	51.9-61.8	43.3-46.4
%$\overline{X}$ relative to the value obtained for homologous RNA	100%	82.71%	79.30%	82.07%	64.67%

Saturation Curves for Filter Bound DNA

Competition in a liquid system, with DNA in excess of the RNA, gives competition values which reflect the relative abundance of the different RNA species present. Competition in a filter bound DNA system, with RNA in excess of the DNA, gives competition values which reflect the relative abundance of the DNA cistrons which code for the different RNA species present.

Saturation and competition curves using filter bound DNA were carried out as described in Materials and Methods. The saturation curve needed to find the minimum amount of labeled RNA to use in competition experiments was generated by adding 0 to 350 ug of labeled RNA to the filter bound DNA. As can be seen from Fig. 7, saturation of the DNA occurs when 210 ug or more of RNA is used. At this point, 0.4 ug of RNA remains bound to the DNA after RNAase treatment. For the competition curves, 220 ug of labeled RNA was used.
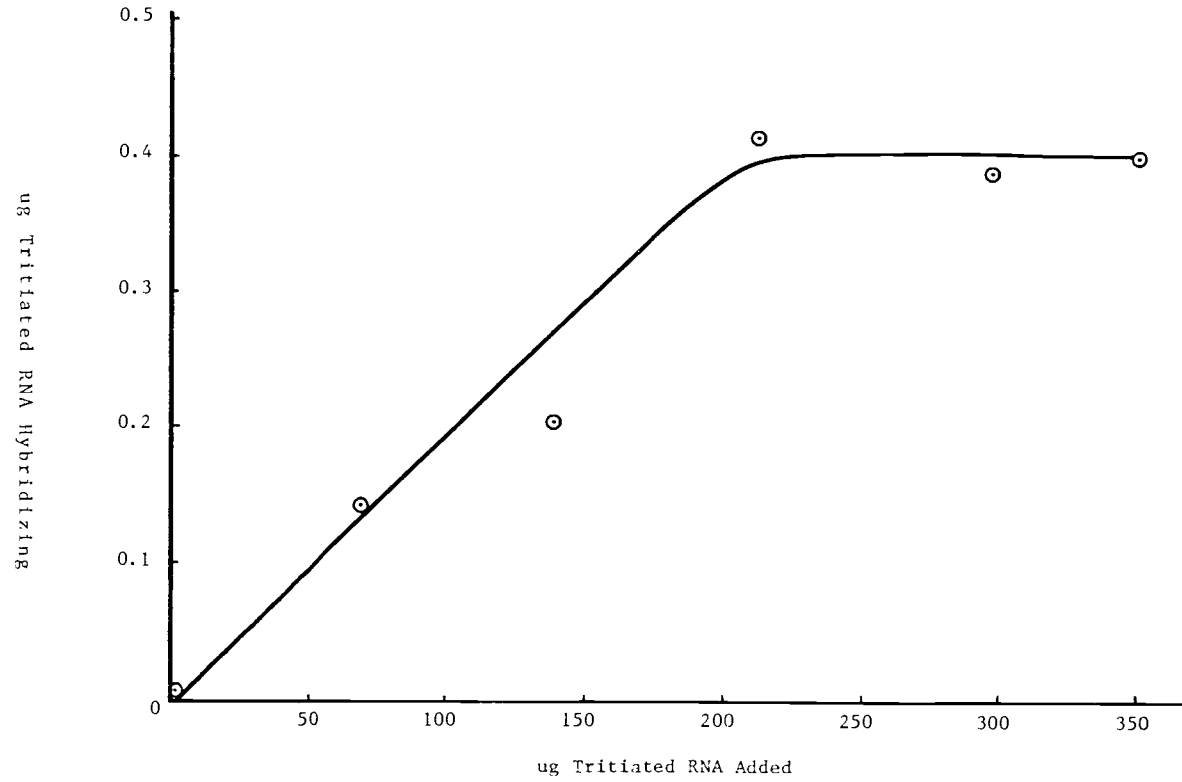
Competition Experiments
Using Filter Bound DNA

Competition curves were run using 220 ug of tritiated HD 109D RNA and 0 to 4000 ug of cold competitor RNA. The

FIGURE 7

Filter Bound DNA Saturation Curve

ug Tritiated RNA Added

amount of labeled RNA hybridized in the absence of any competitor was determined to be 0.43 ug. This was calculated from its specific activity and from the counts remaining on the filter. Background counts, obtained by adding labeled RNA to a blank filter in the hybridization buffer, were negligible. As can be seen from the graph (Fig. 8), homologous competitor RNA gives almost 91% competition, with the maximum competition occurring at 2000 ug or more of competitor RNA. Ribosomal RNA competed to a level of 43% (48% competition relative to that obtained with homologous RNA). The relative competition found for r-RNA in the liquid hybridization system was 65%. This indicates that 48% of the DNA transcription is coding for r-RNA, and this class of RNA comprised 65% of the total RNA species present during the pulse period when the HD 109D is incubated in the casamino acids medium.

The RNA extracted from the HD 109D grown in the S. serpens cell sonicate for both the complete life cycle and for a half a life cycle, competed to levels of 83.9% and 83.7% respectively. The values are essentially the same and show about 92% competition relative to that obtained using homologous RNA, or a relative difference between the two of approximately 8%, compared to the 19% difference between the two found using the hybridization in solution method. This shows that 8% of the DNA being transcribed is producing 19% of the total RNA produced by the HD 109D

FIGURE 8

Competition Experiment Using HI 109D Filter Bound DNA
and 220ug of Labeled HD 109D Total RNA
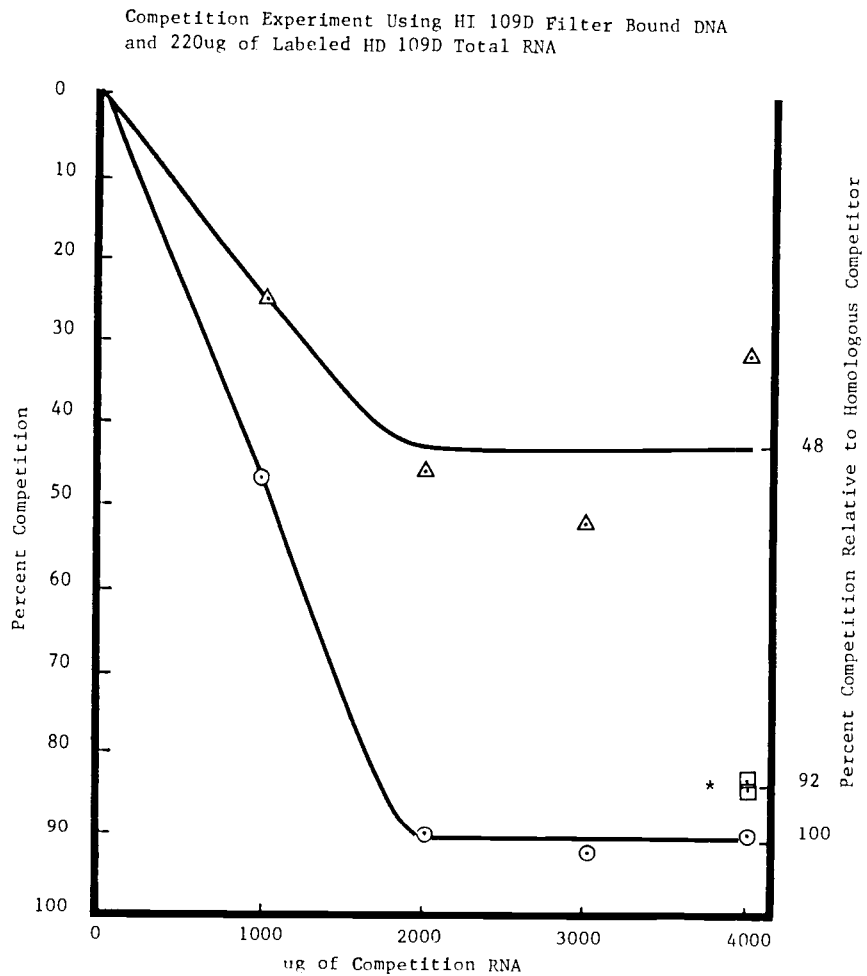
Cold Competitor RNA's Used:

△   HI 109D r-RNA
⊡   HD 109D total RNA from a culture grown to midcycle in host
    cell sonicate (83.7% competition) and a culture grown full-
    cycle in host cell sonicate (83.9% competition).
⊙   HD 109D RNA homologous to the labeled RNA.

*   Raw counts are 2224/sec and 1975/sec for RNA from
    HD 109D grown to mid-cycle in host cell sonicate and
    2219/sec and 1939/sec for RNA from HD 109D grown
    full cycle in host cell sonicate.

incubated in the casamino acids media. The other 44% of the total active DNA, (that not coding for either r-RNA or for the m-RNA produced only during incubation in casamino acids media), is coding for the 16% of the m-RNA which is shared by the other time periods examined.

DISCUSSION

The hybridization/competition system using filter
bound deoxyribonucleic acid (DNA) shows the fraction of DNA
transcribing different ribonucleic acid (RNA) species at
different times (12). The hybridization/competition in
solution shows the relative proportion of the total labeled
RNA which is also produced during the time period from
which the competitor RNA was extracted (that fraction of
the label being excluded from the DNA during competition)
and the relative proportion which is not produced during
the time period from which the competitor was taken (that
fraction of the label hybridizing to the DNA during
competition) (18). There is thus a basic difference in the
information provided by the two systems.

The saturation of filter-bound DNA with labeled RNA
from host-dependent <u>Bdellovibrio bacteriovorus</u> 109(Davis)
(HD 109D) incubated in casamino acids medium (Fig. 7)
showed that about 0.43 ug of labeled RNA was the maximum
which could be hybridized to the filter-bound DNA. The
average amount of DNA remaining on the filter under these
conditions was found to be 9.2 ug (page 27 of Materials and
Methods). The fraction of DNA which binds the labeled RNA
is therefore 0.4 ug RNA/9.2 ug DNA or 4.3%. The
competition experiment using ribosomal RNA (r-RNA) to

compete with the labeled RNA for sites on the filter-bound HI 109D DNA (Fig. 8) showed that the r-RNA replaced 48% of the total possible label which could hybridize with the DNA. Since the total amount of labeled RNA which will bind to the DNA represents 4.3% of the DNA transcribing RNA during the time period the RNA was labeled, and since 48% of this is homologous to r-RNA, 2.1% of the DNA must be coding for r-RNA (48% x 4.3% = 2.1%). Assuming the molecular weight of <u>Bdellovibrio</u> <u>bacteriovorus</u> 109D DNA to be 1.34×10^9 daltons (25), and the molecular weight of the ribosomal RNA to be 2.05×10^6 daltons (17), 2% of the <u>Bdellovibrio</u> DNA should code for 13 ribosomal cistrons (($2\% \times 1.34 \times 10^9)/(2.05 \times 10^6)$). Such calculations must assume that all classes of cell RNA labeled during the 10 minute pulse period have the same specific activity. If for example, the r-RNA had a higher specific activity, competition by r-RNA would displace a disproportionate number of counts and lead to an overestimate of the number of DNA cistrons coding for r-RNA.

Kennell (12) did much the same type of experiment with <u>Escherichia</u> <u>coli</u>. However, he used tritiated r-RNA and was therefore able to directly calculate the fraction of DNA which transcribed r-RNA from the amount of labeled r-RNA which hybridized. His calculations showed that 0.3 % of the <u>E. coli</u> DNA was complementary to r-RNA, and that this would code for 5 r-RNA genes. This is about half the

number of genes that Bdellovibrio bacteriovorus 109D
apparently has to code for r-RNA. Using tritiated total
RNA from the E. coli, Kennell (12) also found that about
10% of the E. coli DNA was actively transcribing RNA, which
is about twice the amount of DNA undergoing transcription
found for the HD 109D incubated in casamino acids medium.
This is really not suprising however, because HD 109D
cannot initiate balanced growth in casamino acids media
(28,32,36) and its rate of metabolism under these
conditions is comparable to that of non-growing E. coli
(9). The total RNA which Kennell used for his experiments
was extracted from E. coli when it was in logarithmic
growth.

The competition experiments carried out in solution
(with DNA in excess of the RNA) showed that cold competitor
r-RNA successfully competed for 65% of the of the possible
sites open to the labeled HD 109D RNA (Fig. 4 and Table 5).
The competition experiments carried out with membrane bound
DNA showed that cold competitor r-RNA successfully competed
for 48% of the possible sites open to the labeled HD 109D
RNA (Fig. 8). In other words, 65% of the total RNA labeled
during the pulse period in casamino acids medium is r-RNA,
and this is produced from 48% of the DNA being transcribed
during this period. Based on the information available
about the use of ribosomes by host-dependent Bdellovibrio,
it seems rather surprising that 65% of the RNA synthesis

was for r-RNA. Hespell et. al. (9) have shown that when HD 109J is suspended in buffer, its proteins and ribosomes undergo rapid and extensive degradation. Suspension of the bdellovibrios in buffer containing amino acids will slow, but will not halt this process. After 20 hours of incubation in buffer containing amino acids, uniformly labeled HD 109J had lost about 32% of the label from its RNA compared to the control. Direct chemical analysis of RNA present in the starved cells showed they contained 32% less RNA than the control. The close correlation of the two methods would indicate little, if any, new synthesis of r-RNA in the amino acid supplemented buffer. However, as stated, this study was done over a period of 20 hours and thus may not reflect the patterns of RNA synthesis and degradation occuring within the first 35 minutes of incubation in casamino acids medium, which is when the HD 109D was labeled for the experiments in this paper.

In another study, Hespell et. al. (7) showed that HD 109J did not synthesize any r-RNA until it was about 40% of the way through a 225 minute developmental cycle in E. coli. The r-RNA present in the bdellovibrios upon entrance into an E. coli cell remained in the cells throughout the entire developmental cycle. Again, due to the difference in experimental conditions, there is no reason to assume that the lack of r-RNA synthesis upon the initial entrance of an HD 109J into a host cell means there cannot be r-RNA

synthesis by the organism at an early stage of its incubation in casamino acids medium.

The competition experiments carried out in the filter-bound DNA system used (1) the pulse labeled RNA, (2) competitor RNA extracted from HD 109D grown through a full development cycle and (3) competitor RNA extracted from HD 109D grown to the mid-point in its developmental cycle. (Ribosomal RNA was also used as competitor and those results have already been discussed.) In this set of experiments, the RNA from HD 109D grown to the mid-point in its life cycle successfully competed for 92% of the sites available to the pulse labeled RNA (Fig.8). This means that 92% of the DNA transcribing RNA during the 10 minute pulse period is also transcribing RNA at the time period in which the competitor RNA was extracted. Since both the labeled and competitor RNA were total RNAs, 48% of the competition is due to the r-RNA in the competitor total RNA (found by competition with cold r-RNA). This leaves 44% (92% - 48%) as messenger RNA (m-RNA) which was transcribed from the same DNA cistrons during the two phases of the life-cycle. The remaining 8% (100% - 92%) of the labeled RNA which successfully competed for sites on the DNA shows the percentage of the DNA transcribing m-RNA during the time of the 10 minute labeling period but not during the time the competitor RNA was extracted. The competition experiments using competitor RNA extracted from HD 109D

grown through its full life-cycle show almost exactly the same results (Fig. 8).

It is not possible to determine from this set of experiments whether or not the 8% of the DNA transcribed during the pulse period but not at the mid-point in the developmental cycle of HD 109D, is the same or different from the 8% which is not transcribed when the HD 109D has completed its life-cycle. However, the number of genes on this portion of DNA which are coding for proteins can be estimated in much the same manner that the number of genes coding for r-RNA were calculated. The amount of DNA involved is 8% of that portion of the DNA which is saturated by labeled HD 109D RNA. Calculating from the saturation curve of filter-bound DNA (Fig. 7), 0.032 ug (0.4 ug x 8%) or 0.35% (0.032 ug/9.2 ug) of the total DNA is coding for m-RNA found only during incubation of HD 109D in casamino acids medium. If one assumes, as Kennell does (12), an average weight of 4.0×10^4 daltons (about 300 amino acids) for the average polypeptide, with three nucleotide pairs (660 daltons/pair) coding for one amino acid, then 4.7×10^6 daltons of DNA (($0.35\% \times 1.34 \times 10^9$)/($5.94 \times 10^5$)) will code for 7 - 8 polypeptides.

The competition experiments carried out in solution (with DNA in excess of the RNA) showed that cold competitor RNA, extracted from HD 109D grown to the mid-point in its developmental cycle, successfully competed for 81% of the

sites available to the labeled RNA (Fig. 4 and Table 5). Since total RNA was used for both the labeled and competitor RNA, 65% of this competition (found by competition with cold r-RNA) is due to the unlabeled r-RNA in the competitor. This leaves 16% (81% - 65%) of the competition due to m-RNA species common to both time periods. The remaining 19% (19% of the total amount of label capable of hybridizing with the DNA) of the labeled RNA which successfully competed for sites on the DNA, is m-RNA transcribed during the 10 minute labeling period but not during the time period from which the cold competitor RNA was extracted. The competition experiments carried out in solution using cold competitor RNA from log-phase HI 109D and from HD 109D grown through a complete development cycle show almost exactly the same results (Fig. 4 and Table 5).

Recalling from the hybridization experiments with the filter-bound DNA (Fig. 8) that 48% of the transcribed DNA is coding for r-RNA, and that 44% is coding for the RNA produced during both the time period at which the HD 109D was labeled and the time period from which the competitor RNA was extracted, then 48% of the transcribed DNA is coding for 65% of the total RNA in the form of r-RNA, and 44% of the transcribed DNA is producing 16% of the m-RNA species found at both the time period in which the HD 109D RNA was labeled, and the time period from which the

competitor RNA was extracted. The 8% of the transcribed DNA in the filter-bound DNA system (Fig. 8) which transcribed m-RNA only during the time period at which the HD 109D was labeled, is therefore coding for the 19% of the total labeled RNA which successfully competed for sites on the DNA in the liquid hybridization system. Since 8% of the transcribed DNA is calculated to code for 7-8 average size polypeptides, and since the 19% of the total RNA it codes for is almost half the m-RNA produced during the pulse period, then 7-8 DNA cistrons are apparently coding for approximately half the m-RNA produced during the time when HD 109D was pulse labeled in casamino acids.

The competition results obtained in the two hybridization systems with the use of the same cold competitor total RNA's, though different for each of the two systems, were nearly identical within each system. It is possible that this is a coincidence. The two competition/hybridization systems used here show the proportions of total RNA being produced at the time period during which the RNA was labeled and the time period from which the competitor RNA was extracted, and the proportion of transcribed DNA producing the RNA at these time periods. These systems give no specific information about which species of RNA are involved, unless a known species of RNA is extracted (r-RNA for example) and used as either labeled RNA or as cold competitor. For example, the

competition/hybridization experiments (carried out in solution and with filter-bound DNA) showed that 65% of the pulse labeled RNA is present as r-RNA produced by 48% of the DNA being transcribed during the pulse period. The other 35% is m-RNA produced by the other 52% of the DNA being transcribed during the pulse period. Of the DNA transcribing m-RNA, 44% is producing 16% of the total RNA as m-RNA during all the time periods tested. The other 19% of the pulse labeled RNA is m-RNA produced only during the pulse period by 8% of the DNA being transcribed at that time. This system does not show whether the DNA retaining labeled RNA after competition with cold RNA extracted from the mid-life cycle of the HD 109D is the same or different from the DNA retaining label after competition with RNA extracted from the organism at the termination of a cycle.

Studies of host-dependent bdellovibrios in host-free media containing amino acids have shown that they can utilize these compounds, primarily glutamate, to synthesize proteins and RNA, but they cannot initiate DNA synthesis or balanced growth (36). Furthermore, under these conditions the host-dependent bdellovibrios rapidly degrade their own proteins and ribosomes (9). Since a basic capacity for energy metabolism is maintained throughout the Bdellovibrio life-cycle, (Bdellovibrio is known to maintain a TCA cycle for example, and cytochromes (25,27,30)), it can therefore be expected that the fraction of the DNA coding for this

energy system will be transcribed throughout the life-cycle of the organism. The proportion of the total RNA out-put this energy system would comprise during the incubation of the Bdellovibrio in casamino acids medium is not known. If it comprises a fairly large proportion of the m-RNA produced under starvation conditions, then the same species of m-RNA are probably involved in the successful competition for DNA sites in the experiments presented in this paper. This would explain why identical sets of competition results were obtained for each of the two different types of competition experiments. It would also leave 8% of the DNA transcribed under these conditions producing almost half of the m-RNA. It is not immediately obvious why casamino acids medium should induce the production of unique species of RNA. It may be that this is the response of the HD 109D to starvation rather than to the medium. Since the Bdellovibrio will degrade its own proteins and r-RNA during starvation (9), the unique class of RNA produced here may be for the synthesis of products to regulate this process.

If, on the other hand, one assumes that the identical competition results produced by the different competitor RNA's are a coincidence, then 36% of the transcribed DNA is coding for m-RNA common to the time periods investigated. This would be for the basic energy system. The other 16% of the transcribed DNA is coding for m-RNA species of which

all are found at the early stage of the organism's life cycle and various others are found at later stages. In this case there would be no species of m-RNA unique to the early stage of the life cycle.

Given the efficient regulation by the Bdellovibrio of its energy metabolism (7,8,16,26), it is not clear what products manufactured during the mid and terminal points of its life-cycle would be of such importance under starvation conditions that they should comprise such a large proportion of the m-RNA. The first explanation of the results is the simplest and therefore the most attractive. However, neither explanation can be adopted or rejected without further experimentation.

CONCLUSION

Interpretation of the data from the experiments presented in this study requires that the following points be kept in mind: 1) Tritiated (5-3H) uracil was introduced into host-dependent Bdellovibrio bacteriovorus 109(Davis) (HD 109D) under conditions of nutritional stress. Ribonucleic acid (RNA) produced during this period, therefore, may not be representative of the types of syntheses normally occuring during growth of the organism. 2) Cold competition RNA not homologous to the labeled RNA was derived from HD 109D grown in a heat treated crude host cell sonicate. It is uncertain how closely RNA synthesis in this system duplicates RNA synthesis of HD 109D invading living host cells. 3) Hybridization/competition experiments under conditions of excess deoxyribonucleic acid (DNA) will give results in terms of total RNA produced. 4) Hybridization/competition experiments with excess RNA will give results in terms of total DNA transcribed.

Hybridization/competition experiments with DNA in excess of the RNA demonstrated that r-RNA comprised 65% of the total RNA produced during the early stage of the life cycle of Bdellovibrio. Of the total RNA produced during this time, 19% is labeled messenger RNA (m-RNA) which

remains after competition with unlabeled total competitor RNA derived from HI 109D in logarithmic growth, and HD 109D grown in host-cell sonicate. The 16% difference in competition between unlabeled ribosomal RNA (r-RNA) and unlabeled total RNA from both host - independent Bdellovibrio bacteriovorus 109(Davis) (HI 109D) and HD 109D is the portion of the total RNA produced as m-RNA during the time of radioactive labeling which is also transcribed at the time the cold competitor total RNA was extracted.
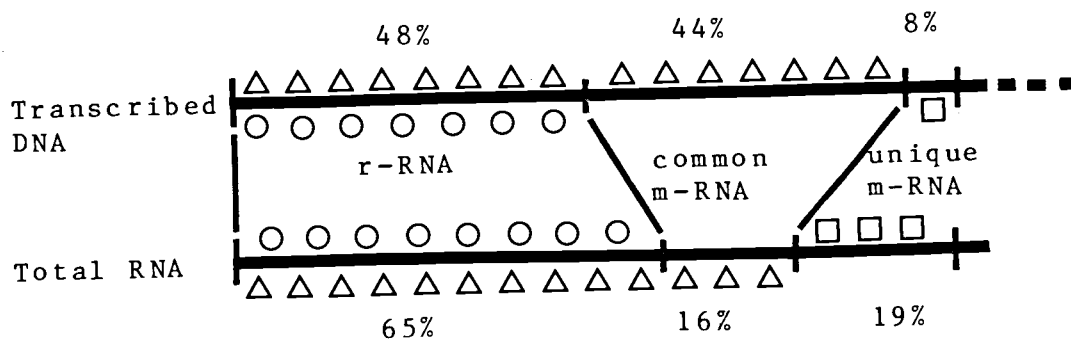
Hybridization/competition experiments with RNA in excess of the DNA demonstrated that 48% of the DNA transcribing RNA during the labeling period was coding for r-RNA. This corresponds to approximately 13 ribosomal cistrons. Of the transcribed DNA, 8% coded for m-RNA which remained hybridized to the DNA after competition with unlabeled total RNA from HD 109D grown in host-cell sonicate. Making certain assumptions about the average size of protein molecules, this amount of DNA probably codes for 7 or 8 polypeptides. The 44% difference in competition between unlabeled r-RNA and unlabeled total RNA from HD 109D grown in host-cell sonicate is the fraction of DNA transcribing m-RNA during the time of radioactive labeling and during the time from which the cold total competitor RNA was extracted.

Taken together, these results show that 48% of the DNA transcribing RNA during the early phase of the life cycle

of HD 109D is producing 65% of the total RNA as r-RNA.   Of
the   transcribed DNA, 44% is producing 16% of the total RNA
as m-RNA common to the early phase in   the   life   cycle   of
Bdellovibrio   and to later stages as well.   The other 8% of
the transcribed DNA is producing 19% of the   total   RNA   as
m-RNA   found   only during the early stage of the life cycle
of the organism (Fig. 9).   In   other   words,   8%   of   the
transcribed   DNA   is producing about half of the m-RNA.   It
cannot be determined from these results whether or not   the
species   of m-RNA resistant to competitor RNA from one time
period in the HD 109D life cycle is the same   or   different
from   that   resistant   to   competitor   RNA   from other time
periods in the life cycle.

FIGURE 9

Relationship of Information Obtained from
the Two Hybridization/Competition Techniques

Hybridization and competition experiments carried out with
RNA in excess of the DNA shows the relative proportions of
DNA being transcribed. The results obtained are represented
by the upper solid line. The remaining DNA not transcribed
during the time of RNA labeling is indicated by the dashed
line and represents 94.8% of the total DNA.

Hybridization and competition experiments carried out with
DNA in excess of the RNA shows the relative proportions of
the total labeled RNA being synthesized. The results
obtained are represented by the lower solid line.

□ - Labeled total RNA obtained from non-growing HD 109D in
casamino acids which hybridized to the DNA during
competition with total unlabeled RNA from HD 109D grown in
host cell sonicate.

△ - Unlabeled total RNA which sucessfully competed with the
labeled RNA for DNA sites.

○ - Unlabeled r-RNA which successfully competed with the
labeled RNA for DNA sites.

Percentages are given relative to the amount of DNA
transcribed (upper line) or amount of RNA produced (lower
line).

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