Application of the Deoxyribonucleic Acid/Ribonucleic Acid Hybridization Technique in *Bdellovibrio* as a Model for Studying Ribonucleic Acid Turnover in Host-Parasite Systems

H. MARK ENGELKING AND RAMON J. SEIDLER*

Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

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The kinetics of host ribonucleic acid (RNA) degradation and its resynthesis into *Bdellovibrio*-specific polyribonucleotides has been studied. The kinetics of RNA turnover was followed during a one-step synchronous growth cycle of *Bdellovibrio* growing within 32P-labeled *Escherichia coli* host cells. The species of labeled RNA present at any given time was ascertained through the specificity of the deoxyribonucleic acid (DNA)/RNA hybridization technique. At near-saturating levels of RNA and at zero time, 7% of the host DNA sequences and only 0.04% of the *Bdellovibrio* DNA became hybridized with 32P-labeled host cell RNA (greater than 99% host specific). At the end of the burst, 98% of the labeled RNA sequences were specific for *Bdellovibrio* DNA. About 74% of the initial labeled host cell RNA became turned over into *Bdellovibrio*-specific sequences. We provide data indicating that host cell ribosomal RNA is assimilated by *Bdellovibrio*. Degradation of host cell RNA occurs in a gradual fashion over most of the *Bdellovibrio* developmental growth cycle. This application of the DNA/RNA hybridization technique and its general concept should be of value in elucidating the kinetics of nucleic acid turnover in other types of host-parasite systems.

*Bdellovibrio* are unique prokaryotes since they invade and multiply within the periplasm of other gram-negative bacteria (17-19). This association seems to involve both a unique physiological dependence for a host chemical not found in complex media as well as a general nutritional dependence for host cell macromolecules. Thus, *Bdellovibrio* deoxyribonucleic acid (DNA) initiation is dependent upon a Pronase- and ribonuclease (RNase)-sensitive host component, whereas other experiments have demonstrated that *Bdellovibrio* elaborate a variety of extracellular hydrolytic enzymes that can degrade host cell proteins, nucleic acids, and murein (3, 4, 6). Recent experiments have focused on the physiological relevancy to *Bdellovibrio* metabolism and nutrition of some of these enzyme activities. Radioactive host cell protein, DNA, and ribonucleic acid (RNA) are degraded by *Bdellovibrio* enzymes, and the products are subsequently assimilated into the macromolecules of growing host-independent bdellovibrios (3). These and other experiments make clear that most classes of host cell macromolecules become digested during the periplasmic sojourn of the *Bdellovibrio*.

In a series of elegant experiments, Matin and Rittenberg used CsCl equilibrium centrifugation to follow the *Bdellovibrio*-induced degradation and turnover of host cell DNA (8). Physical properties of the two DNA species of different guanine plus cytosine base compositions provided the necessary basis for discriminating *Bdellovibrio* from host cell DNA. In the present study, we used a host-dependent *Bdellovibrio* strain to quantitatively examine the kinetics of host RNA degradation and turnover, using the polynucleotide specificity as detected in DNA/RNA reassociation experiments. In this manner it becomes possible to demonstrate the kinetics of turnover of this host cell nucleic acid during various phases of the synchronized *Bdellovibrio* life cycle.

MATERIALS AND METHODS

Organisms and media. *E. coli* CSH42 was used to prepare [methyl-3H]thymidine-labeled DNA and 32P-labeled host cell nucleic acids for DNA/RNA hybridization studies. The *E. coli* was grown in modified CM broth (12) supplemented with 1% vitamin-free Casamino Acids (Difco), 1% glucose, 5 μg of

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thymidine per ml, and 0.5 μg of thiamine per ml.

Host-independent *B. bacteriovorus* 109D was grown in PYE broth (15). Host-dependent *B. bacteriovorus* 109D was grown in PYE/10 broth containing 3 mM Ca**+** and 2 mM Mg**+**. PYE/10 is 1 part PYE and 9 parts 1 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.5.

**Synchronous growth conditions and PFU enumeration.** Optimum cultural and physiological conditions for achieving a homogeneous population of *Bdellovibrio*, which is necessary for synchronous development, have been described (2). In this study, infections were initiated in PYE/10 broth containing calcium and magnesium as indicated above. The multiplicity of infection was approximately 2, and the initial *Bdellovibrio* titer was in the range of 10^8 to 2 × 10^9 plaque-forming units (PFU)/ml. All cultures were incubated at 30°C.

PFU were enumerated by the double agar overlay method as previously described (16).

**Preparation of DNA for hybridization studies.** *E. coli* CSH42 was grown in CM broth containing 1 μCi of [methyl-3H]thymine per ml. Host-independent *B. bacteriovorus* 109D was grown in PYE broth containing 0.5 μCi of [methyl-3H]thymidine per ml. The DNA was extracted and purified by conventional techniques (9, 15). Unlabeled DNA from both cultures was prepared and added to the labeled DNA preparations for adjusting specific activities. DNA purity was assessed by 260/280 ratios (1.90 or greater) and 260/230 ratios (2.25 or greater). Samples of the DNA were melted for thermal midpoint calculations in the hybridization buffers. The DNA exhibited a hyperchromic shift of 35 to 37% and did not show any optical shifts at lower temperatures that would indicate RNA contamination.

**Preparation of labeled RNA for hybridization experiments.** RNA of the *E. coli* host was labeled by growing the culture for 12 h in 250 ml of CM broth containing 40 μCi of [3P] per ml as inorganic phosphate. The culture medium used was limiting in phosphate concentration. It contained 20 μg/ml as KH₂PO₄ and was 20 mM in Tris buffer, pH 7.5. These ingredients were added to CM medium.

Total cell RNA was obtained by the extraction technique of Moore and McCarthy (12). This procedure involves a two-stage treatment with deoxyribonuclease (DNase; Worthington, electromechanically pure, 10 μg/ml). Cells were thawed in 0.01 M Tris, pH 7.2, containing 0.01 M KCl and 0.01 M MgCl₂, DNase was added, and the cells were disrupted in a French pressure cell at 15,000 lb/in². The second DNase treatment (1 h at 37°C) occurred after the first phenol, Sevag, and ethanol precipitation. Such bulk RNA preparations typically contain about 80% ribosomal RNA (rRNA), 20% transfer RNA, and some 2.5% messenger RNA (mRNA) (7).

The purified [3P]RNA was tested for purity by several means before hybridizations were carried out. The labeled RNA was hydrolyzed to the following levels: 99.2% hydrolysis after 40 min at 95°C in 5% trichloroacetic acid; 97.9% hydrolyzed with pancreatic RNase (50 μg/ml for 30 min at 37°C); and 98.4% hydrolysis in 0.1 N NaOH for 18 h at 37°C. The slightly higher RNase counts probably represent a pancreatic RNase-resistant core RNA. The trichloroacetic acid treatment indicates that about 0.8% of the counts represent non-nucleic acid material. If this figure is subtracted from the NaOH residual, about 1% of the material could be contaminating DNA. Such trace amounts of DNA will not significantly influence the observed DNA/RNA hybridization levels. This is based on the observed 0.2% saturation of the DNA for 1 μg of input bulk cell RNA (Table 1). This level of RNA hybridized does not differ from that expected from the 0.8 μg of rRNA input levels observed by others (1, 12).

**DNA/RNA hybridization experiments.** *E. coli* CSH42 [3P]-labeled host cells and *Bdellovibrio* were prepared for a synchronous one-step growth experiment (2). Samples (40 ml) of the culture were removed at hourly intervals for 6 h. These samples for RNA extraction were centrifuged (15,000 × g for 15 min), washed with saline-ethylenedianiaminetetraacetic acid (9), and frozen. Bulk RNA from the *Bdellovibrio*-infected host cell suspension was extracted and used in the hybridization experiments.

DNA/RNA hybridization studies were performed by using the membrane filter technique of Gillespie and Spiegelman as modified by McCarthy and McConaughy (5, 11). We have modified the original procedures by using dual labels ([3H]-labeled filter-bound DNA and [3P]RNA in solution) and dimethyl sulfoxide in the buffer to lower the optimum incubation temperature during renaturation and inhibit DNA leaching from filters. The buffer used consisted of double-strength standard saline citrate buffer (2 × SSC; 10) and 40% dimethyl sulfoxide. Hybridization was carried out at 25°C below the midpoint of the thermal melting curve as observed in this buffer (38°C).

**Measurement of radioactivity.** Radioactivity was

| Amt of E. coli RNA in hybridization mixture (μg) | E. coli RNA (μg) bound to: |
|-------------------------------------------------|----------------------------|
| 10 μg of E. coli DNA                            | 10 μg of Bdellovibrio DNA |
| [3P]E. coli DNA                                 | Blank filters             |
|-------------------------------------------------|----------------------------|
| 1                                               | 0.020                      | 0.004                     | 0.001                     |
| 5                                               | 0.096                      |                           |                           |
| 10                                              | 0.320                      |                           |                           |
| 20                                              | 0.600                      |                           |                           |
| 30                                              | 0.860                      |                           |                           |
| 50                                              | 0.936                      |                           |                           |
| 100                                             | 0.880                      |                           |                           |
| 50                                              | 0.936                      |                           |                           |
| 50                                              |                           | 0.004                     | 0.001                     |

* Values are the average of three separate hybridizations. The mixture refers to the 0.5 ml of the hybridization buffer, 2 × SSC and 40% dimethyl sulfoxide. The specific activity of the *E. coli* [3P]-labeled RNA was 70,000 counts/min per μg and *Bdellovibrio* [3H]-labeled DNA was 254 counts/min per μg.
RESULTS AND DISCUSSION

The choice in this study to follow RNA degradation and turnover by a \(^{32}\)PO\(_4\) label was based on the report of a unique nucleotide metabolism in *Bdellovibrio*. The *Bdellovibrio* preferentially use phosphate from the host cell and not from the culture medium. This utilization involves assimilation of nucleic acid precursors in the form of the nucleoside monophosphate (14). Thus the presently described approach would probably not be applicable to other microbial systems that would need a \(^{14}\)C or \(^{3}H\) label in the pyrimidine uracil.

Preliminary experiments were necessary to define the specificity of the DNA/RNA hybridization assay. Only 0.002% of the 50 \(\mu\)g of labeled *E. coli* RNA bound to blank filters (Table 1). A comparably low value was obtained from *Bdellovibrio* RNA. Good species specificity in the hybridization assay was also achieved since only 0.5% of the *E. coli* hybridizable RNA would form RNase stable hybrids with *Bdellovibrio* DNA. The reciprocal reaction ( *Bdellovibrio* RNA to *E. coli* DNA) is documented in the 6-h time sample of Table 2. This reaction was at least 98% specific (0.012/0.564 \(\mu\)g).

| Table 2. Specificity and extent of RNA reassociation during *Bdellovibrio* infection of \(^{32}\)P-labeled *E. coli* host cells* |
|---------------------------------------------------------------|
| **RNA sample time**                                            | **\(\mu\)g of RNA bound/10 \(\mu\)g of DNA** |
| **(h)**            | **Trial I** | **Trial II** | **Avg** | **Change/h (avg)** |
| *Bdellovibrio* DNA on filter       |             |             |         |                   |
| 0                   | 0.003       | 0.005       | 0.004   |                    |
| 1                   | 0.021       | 0.022       | 0.022   | +0.018             |
| 2                   | 0.021       | 0.017       | 0.019   | -0.003             |
| 3                   | 0.036       | 0.058       | 0.047   | +0.028             |
| 4                   | 0.350       | 0.400       | 0.375   | +0.328             |
| 5                   | 0.659       | 0.684       | 0.671   | +0.296             |
| 6                   | 0.577       | 0.551       | 0.564   | -0.107             |
| *E. coli* DNA on filter       |             |             |         |                   |
| 0                   | 0.625       | 0.760       | 0.697   |                    |
| 1                   | 0.301       | 0.389       | 0.345   | -0.352             |
| 2                   | 0.255       | 0.300       | 0.282   | -0.063             |
| 3                   | 0.252       | 0.177       | 0.215   | -0.067             |
| 4                   | 0.194       | 0.118       | 0.156   | -0.059             |
| 5                   | 0.154       | 0.122       | 0.138   | -0.018             |
| 6                   | 0.012       | 0.012       | 0.012   | -0.126             |

*TriPLICATE hybridization samples were run at each time period with both types of filters. Trials I and II refer to separate experiments.

An input RNA level was chosen for the experimental hybridization reactions that would both detect a very large percentage of the different species of RNA (including mRNA) and at the same time be responsive to changes in the composition of the bulk cell RNA. Near saturation of hybridizable *E. coli* RNA was achieved at about 30 \(\mu\)g of RNA (Table 1). A comparable level of optimum RNA concentration is also achieved when *Bdellovibrio* DNA/RNA competition experiments are studied (15). About 8 to 9% of the *E. coli* DNA becomes saturated with 30 to 100 \(\mu\)g of RNA. This level represents near saturation of the transcribed regions since it approaches the 9 to 10% maximum saturation reported for long-labeled *E. coli* RNA (7).

A total of 50 \(\mu\)g of RNA was used for the experimental series of hybridizations (Table 2, Fig. 1). This resulted in about 26 \(\mu\)g of host RNA and 24 \(\mu\)g of *Bdellovibrio* RNA in the zero-time sample. These concentrations are based on the change in specific activity of the RNA after
addition of *Bdellovibrio* (66,000 versus 35,000 counts/min per μg; Table 2). The 26-μg input level of host RNA had the desirable effect of a slight reduction in the DNA saturation to about 7% (Table 2). This makes the hybridization assay more sensitive in detecting changes in the concentration of host RNA during the infection cycle.

The kinetics of RNA saturation to *E. coli* DNA (Table 1) demonstrates a linear relationship, up to 30 μg between the amount of RNA hybridized and the amount of RNA placed into the reaction vial. This relationship can be extrapolated to the experimental system (Table 2, Fig. 1) to permit an estimate of the concentration of host RNA present in the bulk extract at the various stages of infection. Such an estimate is based on the assumption that all classes of host RNA undergo comparable rates of degradation; i.e., there is no differential specificity in the RNase activities. This assumption seems justified since the level of DNA saturated by the host RNA in the extract continues to remain significantly above that expected by rRNA and transfer RNA alone through the 5-h time sample.

The information in Table 2 summarizes the extent of RNA reassociation to both *E. coli* and *Bdellovibrio* DNA filters during a synchronous *Bdellovibrio* growth cycle. Hybridization experiments were run on RNA extracted at seven periods during the synchronized infection of 32P-labeled *E. coli* host cells. At zero time all the RNA label was contained in the host polyribonucleotides, and this is reflected in the 99.5% specificity of the RNA reassociating with the *E. coli* DNA. Reassociation by 0.7 μg of *E. coli* RNA per 10 μg of DNA indicated that 7% of the DNA sites were saturated. Thus, the label is in both rRNA as well as in mRNA species since ribosomal DNA cistrons of bacteria do not comprise more than 0.4% of the genome (7, 13). The presence of label in both classes of RNA was desirable since higher saturation levels and greater counts could be obtained on the filters. At the end of the infection (6 h) over 98% of the initial *E. coli* RNA species were lost from the whole-cell RNA extract. The label then appeared in the form of *Bdellovibrio*-specific polyribonucleotides.

Previous studies on the microscopic events of the *Bdellovibrio* life cycle have demonstrated that the host cytoplasm undergoes disorganization and the host protoplast diminishes in size (16, 17). It has been assumed that these observations indicate a dissolution of the host cell ribosomes, with the implication that these particles serve as protein and nucleic acid sources for *Bdellovibrio* growth. Since the host cell can provide all the necessary nutrients for *Bdellovibrio* multiplication (a burst of five to six *Bdellovibrio* progeny [16]), it appears that turnover of host ribosomal particles would have to occur to supply sufficient metabolites for *Bdellovibrio* growth. The present hybridization data can be used to demonstrate that host rRNA is indeed degraded and subsequently assimilated by *Bdellovibrio*.

The only parameter affecting the amount of RNA hybridized to *E. coli* DNA is the concentration of host RNA present in the extracts. Due to the linear relationship between the amount of host RNA present and the amount of hybrid obtained (Table 1), it is possible to calculate the degradation (disappearance) of the host cell RNA in the 50 μg used for hybridization. As an example, the 5-h sample (Table 2) indicates that about 0.14 μg of *E. coli* RNA hybridized to 10 μg of *E. coli* DNA. This level would be expected from slightly more than 5 μg of *E. coli* RNA (Table 1). The zero-time sample contained about 26 μg of *E. coli* RNA based on both hybridization saturation levels (Table 1) and on changes in specific activity of the RNA preparations (Table 2). There was then a fivefold decrease in the *E. coli* RNA, indicating that degradation of about 80% of the initial host RNA had occurred. Since about 80% of the long-labeled *E. coli* RNA is in rRNA (7), considerable host RNA had been assimilated into de novo *Bdellovibrio* RNA.

The kinetics of PFU titer increase and the changing pattern of RNA hybridization specificity are displayed in Fig. 1. By the second hybridization sample at 1 h, there was already some destruction of host cell RNA and a resynthesis into *Bdellovibrio*-specific polyribonucleotides. Our previous studies on the synchronous growth of *Bdellovibrio* have shown that the substrate organism is penetrated within the first 20 to 40 min (16). Thus, soon after penetration is completed, there is a measurable turnover of host cell RNA. The curve demonstrating RNA hybridizing to *Bdellovibrio* DNA was generated by assuming that the newly synthesized *Bdellovibrio* RNA was of the same specific activity as the bulk host RNA in the extract. This assumption may be in error during the very early infection stages until *Bdellovibrio* pools of preexisting cold RNA become exhausted.

We have previously demonstrated that *Bdellovibrio* produce extracellular DNase and RNase activities that can hydrolyze the corre-
sponding host cell macromolecules (3). We presume that the RNase(s) is responsible for the loss of host cell polyribonucleotides from the present hybridization reactions.

The greatest amount of *Bdellovibrio* RNA assimilation occurs during the 3rd h of infection. This corresponds to the active period of *Bdellovibrio* mass increase, which is microscopically observable during this period (16). By the 4th h about 60% of the *Bdellovibrio* have completed the life cycle and left the host cell. Between the 5th and 6th h there is a final 10-fold drop in the last 20% of host-specific RNA. This last decrease occurs after the burst is almost complete and likely indicates a loss into the culture supernatant of the remaining RNA from the ghosted host cells.

During the period of 1 to 4 h there is a continuous and incremental drop of about 10% per h from the initial amount of labeled RNA that remains hybridizable to *E. coli* DNA (Fig. 1, Table 2). Also, since the host RNA is degraded over the entire life cycle, the *Bdellovibrio* are provided with a constant pool of RNA precursors that remain within the infection complex. This aspect of degradation of host cell nucleic acids is analogous to the process reported for *Bdellovibrio*-induced host cell DNA degradation (8).

The 6-h hybridization sample demonstrates a decrease in the level of RNA hybridizing to *Bdellovibrio* DNA (Table 2). This could merely represent a fortuitous result of experimental deviation. However, this lowering of the amount of DNA saturated might also be a reflection of mRNA turnover in *Bdellovibrio*, with a larger fraction of the label reappearing in rRNA sequences.

Table 3 shows the specific activities of the purified RNA that was bound by hybridization. The specific activities can be used to calculate the percentage of host cell RNA turnover and may serve as an indication of net RNA gain or loss from the existing pool of radioactive RNA. The percentage of turnover was derived from the initial and final specific activities. This is possible since the hybridization experiments (Table 2) illustrate that the initial and final RNA preparations were composed of polyribonucleotides from only one of the microbial components. Such calculations indicate that about 74% of the original *E. coli* counts (35,000 counts/min per μg) subsequently appear as *Bdellovibrio*-specific polyribonucleotides (26,000 counts/min per μg). This efficiency of turnover is comparable to that observed for host cell DNA (8).
RNA would contribute to a further net reduction in the specific activity as the host RNA is lost from the infection centers. At present no information is available concerning the possible utilization by Bdellovibrio of host cell ribonucleoside monophosphates for macromolecules other than RNA. The 74% calculated turnover figure, therefore, represents a minimum for the assimilation of host cell RNA.

The present experiments reveal the feasibility and usefulness of the DNA hybridization technique for following the kinetics of nucleic acid turnover in cultures containing more than a single cellular species. However, it is essential to carefully design the hybridization experiments so as to maximize the sensitivity to changes in the nucleic acid composition as development of the cellular components proceed. With the obvious modification, the described procedure is amenable for following turnover of polydeoxynucleotides as well. This technique should allow one to study nucleic acid turnover in a variety of multicomponent systems, including viruses or bacteriophage and their hosts or eucaryotic intracellular parasites such as Rickettsia, Chlamydia, and protozoa.

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