Fermentation Process for Double-Stranded Ribonucleic Acid, an Interferon Inducer

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Double-stranded ribonucleic acid (ds-RNA) isolated from Escherichia coli infected with bacteriophage MS2 is a potent interferon inducer. High levels of ds-RNA are formed in nonpermissive cells infected with MU9, an amber coat protein mutant of MS2. This mutant has been used to develop a process for large-scale ds-RNA production. Preparation of quantities of MU9 lysate sufficient for ds-RNA production in fermentors is described. Over 300 μg of ds-RNA/ml can be accumulated after MU9 infection of cultures grown to high density in corn steep liquor medium. This is approximately 300 times the amount of ds-RNA made by MS2 infection of cells grown in tryptone medium. Maximum ds-RNA formation requires only 3 hr. The ds-RNA is stable and remains inside nonaerated cells for at least 17 hr.

Double-stranded ribonucleic acid (ds-RNA) isolated from male Escherichia coli infected with the RNA bacteriophage, MS2 (2), is a potent inducer of interferon in cultured rabbit kidney cells and in the whole animal (4). However, recovery of ds-RNA from cells infected with MS2 is inefficient and unreliable. Cultures infected after growth to optimum cell densities in tryptone medium produce only 1 μg of ds-RNA/ml. The cultures must be chilled and the cells harvested rapidly prior to lysis, which begins 90 to 120 min after infection.

Amber nonpolar coat protein mutants of RNA bacteriophage, originally isolated from bacteriophage f2 by Zinder and Cooper (20), are far superior to MS2 for ds-RNA production. These mutants produce extremely low levels of a short coat protein fragment during growth in nonpermissive (Su−) cells (16, 18). In the absence of the regulatory influence of functional coat protein (8, 11, 16, 18), abnormally high levels of RNA synthetase and replicative intermediates (containing ds-RNA) are formed. In addition, because coat protein is required for cell lysis (21), nonpermissive cells infected with these mutants do not lyse and ds-RNA remains inside the cells (8).

Production of ds-RNA by an MS2 mutant, MU9, will be described. This MU9 ds-RNA has been isolated by the procedure of Birnbaum and Lampson (U.S. Patent 3,582,468, 1971), and found to be as effective for interferon induction as MS2 ds-RNA (12).

MATERIALS AND METHODS

Organisms. Escherichia coli K90 contains a suppressor of amber mutations (5), Su-1, that inserts serine in growing polypeptide chains at sites corresponding to UAG codons in messenger RNA (19). E. coli Hfr 3000 (Su−), does not contain an amber codon suppressor. The wild-type RNA bacteriophage MS2, originally isolated by A. J. Clark, propagates in both Hfr 3000 and K90. Mutant bacteriophage MU9, isolated by C. I. Davern, has a nonpolar amber mutation (8, 16) in the codon corresponding to the 70th amino acid (glutamine) from the N-terminus of the MS2 coat protein (7); mature virions are not formed in the Su− strain, and the infected culture does not lyse. The inhibition of growth of Hfr 3000 after infection with MU9 is shown in Fig. 1. Observation by phase microscopy revealed that the optical density (OD) increase after infection is the result of a marked increase in cell volume and not of continued cell division.

Medium. Tryptone broth agar (9), used for bacteriophage titration and preparation of soft agar overlay lysates (1), contained (per liter) 10 g of tryptone, 1 g of yeast extract, 8 g of sodium chloride, 0.3 g of calcium chloride-dihydrate, 1 g of dextrose, and 15 g of agar.

Liquid cultures for preparation of lysates and production of ds-RNA were grown at 37°C (unless otherwise specified) either in tryptone broth without glucose or in corn steep medium (15). Cultures were incubated in 250-ml flasks at 220 rev/min on a rotary shaker with a 2-inch (ca. 5.1 cm) displacement. Corn steep medium was prepared by diluting fresh,
well-mixed corn steep liquor (Corn Products, New York) with an equal volume of distilled water and adjusting the pH to 7.0 with solid sodium hydroxide. After centrifugation, the supernatant fluid was diluted to the desired final concentration (v/v) with distilled water, brought to a boil, cooled, clarified by centrifugation, and sterilized for 30 min at 121°C.

ds-RNA assay. Ribonuclease-resistant acid-precipitable RNA was assayed in infected and uninfected samples. The net amount present after virus infection was defined as double-stranded viral RNA. Pelleted cells equivalent to 27 mg of cell dry weight were assayed immediately or stored at -20°C. Pellets resuspended in 2.5 ml of TE buffer [0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 0.001 M ethylenediaminetetraacetic acid (EDTA), pH 7.8] were incubated for 20 min at room temperature with one-tenth volume of a fresh solution of lysozyme (1 mg/ml). Lysis was completed by addition of 0.05 ml of 10% sodium dodecyl sulfate. After clarification, the tubes were chilled and nucleic acids were precipitated by addition of 5.5 ml of ethanol at -20°C and 0.025 ml of 10 × SSC (SSC = 0.1 M sodium chloride, 0.015 M sodium citrate, pH 7.0). Stoppered tubes were shaken and chilled for 10 min. The precipitate, collected by centrifugation, was resuspended in SSC, and single-stranded RNA was digested by 12.5 µg of pancreatic ribonuclease (Worthington Biochem., Freehold, N.J.) per ml at room temperature for 45 min. Acid-soluble digested nucleic acids were removed with cold 5% trichloroacetic acid. The precipitate was washed with 5% cold trichloroacetic acid, redissolved in 2.5 ml of SSC, and clarified by centrifugation. Samples were assayed by the colorimetric orcinol reaction (10) using reagent grade D-ribose as a standard. In each experiment, values were corrected for the orcinol reaction of an equal mass of uninfected cells.

Determination of cell mass. Cell mass was calculated from optical density measured in a Klett-Summerson colorimeter at 660 nm. Infection of Hfr 3000 by MU9 had no effect on the ratio of dry weight to OD (1.7 mg/OD unit).

RESULTS

Production of MU9 lysates. The quantities of MU9 lysate required for large-scale production of ds-RNA in Hfr 3000 must be generated by stepwise propagation in strain K90. Reversion of MU9 to wild type occurs at a high rate (10^{-4} to 2 × 10^{-4} per replication cycle) and the number of infective phage produced by cells infected with MU9 is only 10% the number of infective phage produced by an MS2 infection. As a result, uncontrolled propagation by infection of young cultures at a low multiplicity of infection leads to rapid enrichment of MS2 in the bacteriophage population.

The amount of MS2 enrichment during single-step propagation of MU9 in liquid culture depends primarily on the multiplicity of infection (virus-cell). Infection at a multiplicity of at least 20 is required to prevent MS2 enrichment (Table 1). Culture density, growth conditions, and medium have little effect on lysate quality as long as cells are growing exponentially with a generation time of 25 to 45 min when infected.

Lysates are initiated from single MU9 plaques collected 4 to 5 hr after plating and eluted overnight at 4°C in 5 ml of tryptone broth. Eluates contain between 10^4 and 10^7 phage/ml. This number is too low for subsequent propagation of MU9 in liquid culture. Therefore, secondary lysates are prepared by soft agar overlay. MU9 increases a millionfold, whereas the percent MS2 increases only two- to threefold. Maximum MU9 titers are obtained by plating 10^4 to 5 × 10^4 MU9 with 10^4 exponentially growing K90 cells. After 6 to 7 hr at 37°C, between 10^12 and 10^13 progeny MU9 are recovered from a plate by elution. One-half of the plate lysates initiated with 0.1 ml of different single-plaque eluates contained no more than 0.05% MS2 and one-fifth contained at most 0.03%.

Subsequent scale up by a series of four
ds-RNA theoretically, addition not RNA when nature and eluates quantities Lysates need of single-step liquid lysates, with a 50-fold increase of MU9 and at most a twofold increase of MS2 fraction at each step, generates large quantities of lysate with less than 1% MS2. Lysates need not be clarified at any stage, and addition of EDTA, lysozyme, or chloroform is not required to facilitate cell lysis. Plaque eluates and plate lysates may be subdivided and stored at −10 C for 6 months to a year without detectable loss of infectivity. Lysates prepared in fermentors and then cooled may be stored temporarily without agitation; the titer remained constant for at least 17 hr when a lysate was cooled to 15 C, and even at 28 C 80% of the MU9 were still infective. Lysates can be stored for several weeks at 4 C if chloroform is added, and chloroform removal is not necessary prior to infection with lysate volumes as high as 2.5% of the total volume, providing none of the solvent phase is transferred.

Production of double-stranded RNA. MU9 ds-RNA is produced in Hfr 3000. Cultures are infected with an average of 6 MU9/cell so that, theoretically, less than 0.3% of the cells escape infection. MU9 lysates containing more than 1% MS2 are not used to initiate ds-RNA formation. Even at this MS2 level, 6% of the Hfr 3000 cells could lyse after infection of the culture with 6 virus/cell. Cells grown in tryptone broth produce the maximum amount of ds-RNA when infected at a density of 5 × 10⁸ cells/ml; after 1 hr, 4 to 5% of the cell dry weight is ds-RNA (Fig. 2). Figure 3 shows that most of the ds-RNA is formed by the third hour, and at this time accounts for 8 to 9% of the dry weight.

The optimum temperature for MU9 ds-RNA

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**Table 1. MS2 enrichment in single-step lysate production**

| Virus per cell | Percent MS2 | Cells per ml | Growth conditions | Harvested lysate | MS2 enrichment |
|---------------|-------------|--------------|-------------------|----------------|---------------|
|               | (×10⁸)     | Medium       | Vessel, volume*a  | PFU* per ml    | PFU per cell  | Percent MS2  |
| 5             | 0.03       | 5 × 10⁸      | Tryptone          | (1)            | 12.0          | 2,400        | 0.13         | 4.3          |
| 5             | 0.08       | 6 × 10⁸      | 5% Corn steep     | (1)            | 4.5           | 420          | 0.31         | 4.0          |
| 5             | 0.08       | 10⁸         | 5% Corn steep     | (1)            | 12.0          | 1,200        | 0.27         | 3.4          |
| 5             | 0.08       | 2 × 10⁸      | 5% Corn steep     | (1)            | 8.0           | 400          | 0.71         | 9.0          |
| 10            | 0.03       | 2 × 10⁸      | Tryptone          | (1)            | 3.8           | 1,900        | 0.09         | 3.0          |
| 10            | 0.03       | 5 × 10⁸      | Tryptone          | (1)            | 3.3           | 660          | 0.09         | 3.0          |
| 10            | 0.05       | 10⁸         | 5% Corn steep     | (2)            | 8.4           | 840          | 0.68         | 13.6         |
| 10            | 0.05       | 10⁸         | 5% Corn steep     | (3)            | 8.6           | 860          | 0.22         | 4.4          |
| 15            | 0.03       | 2 × 10⁸      | Tryptone          | (1)            | 1.2           | 600          | 0.08         | 2.6          |
| 20            | 0.03       | 5 × 10⁸      | Tryptone          | (1)            | 4.8           | 960          | 0.06         | 2.0          |
| 20            | 0.06       | 7 × 10⁸      | Tryptone          | (3)            | 9.4           | 3,400        | 0.08         | 1.3          |
| 20            | 0.06       | 5 × 10⁸      | 8.5% Corn steep   | (3)            | 6.1           | 1,220        | 0.08         | 1.3          |
| 20            | 0.06       | 4 × 10⁸      | 8.5% Corn steep   | (3)            | 13.0          | 330          | 0.09         | 1.5          |
| 50            | 0.03       | 5 × 10⁸      | Tryptone          | (1)            | 7.3           | 1,460        | 0.06         | 2.0          |
| 100           | 0.03       | 5 × 10⁸      | Tryptone          | (1)            | 4.6           | 920          | 0.04         | 1.3          |

* (1) 250-ml Erlenmeyer flask, 50 ml of medium; (2) 250-ml baffled flask, 50 ml of medium; (3) 250-ml baffled flask, 20 ml of medium.

* Plaque-forming units.
formation is 37°C (Table 2). Temperature during growth of cells prior to infection has no effect on the amount of ds-RNA produced. Shifts from 33 to 37°C at various times after infection revealed that the duration of ds-RNA formation is affected more than the rate in this temperature range. Cultures shifted at 120 min formed 94% the amount of ds-RNA in control cultures incubated at 37°C throughout the 180 min incubation period. By comparison, cultures not shifted from 33°C made only 75% of the control level.

Tryptone broth cultures with \(5 \times 10^8\) cells/ml would enter stationary phase within 1 to 1.5 generations if not infected. Much higher cell yields are obtained by growth in an aqueous solution of corn steep liquor (Fig. 4). Final cell number is greatest in corn steep liquor concentrations between 8 and 10% and is 7 to 8 times the number produced in tryptone broth. Growth without a lag to maximum density required inoculation with a logarithmic culture grown in the same medium (Fig. 5).

High levels of MU9 ds-RNA are formed after infection of vigorously aerated (20 ml of medium in a baffled flask) corn steep liquor cultures at densities above \(5 \times 10^8\) ml (Table 2). Temperature constant after infection of Hfr 3000.

* Temperature shifts during incubation after infection. The ds-RNA was measured 3 hr after infection of cells grown to \(5 \times 10^8\) ml in tryptone medium with 6 MU9/cell.

![Graph](https://example.com/graph.png)

**Fig. 3.** Accumulation of ds-RNA after MU9 infection of cells grown in tryptone medium to \(5 \times 10^8\) cells/ml at time of infection with 6 MU9/cell.

![Graph](https://example.com/graph.png)

**Fig. 4.** Effect of corn steep liquor medium on cell number. Cell number and culture dry weight were derived from optical density measured 10 hr after inoculation with logarithmic-phase cells growing in 5% corn steep liquor.

![Graph](https://example.com/graph.png)

**Fig. 5.** Effect of corn steep liquor concentration on cell number. Cell number and culture dry weight were derived from optical density measured 10 hr after inoculation with logarithmic-phase cells growing in 5% corn steep liquor.

3). Efficient production after infection at densities above \(4 \times 10^8\) ml requires initiation of growth with actively growing cells. Corn steep liquor concentrations between 8 and 9% support maximum production of ds-RNA by cultures infected at \(8 \times 10^8\) /ml (Fig. 6), and a culture density of \(8 \times 10^8\) to \(10 \times 10^8\) cells/ml at infection is optimum for production in 8.5% corn steep liquor (Fig. 7). The maximum observed yield of 280 mg/liter represents over 4%
of the cell dry weight. Double-stranded RNA does not increase after the third hour in these cultures. However, infection with 12 or 24 virus per cell instead of 6 increases the ds-RNA level to 5% of the dry weight, or 320 mg/liter.

Control of accidental infection. Propagation of MS2 after accidental infection by MS2 prior to intended MU9 infection can be controlled by addition of citric acid to corn steep liquor medium (Table 4). If an equivalent concentration of calcium or magnesium chloride (13) is added at the time of MU9 addition, infection occurs, and normal amounts of virus or ds-RNA are formed. Excessive calcium spuriously elevates the ds-RNA assays by inhibiting digestion of cellular RNA.

FIG. 5. Effect of inoculum on growth rate and final cell density in 5% corn steep liquor. Stationary-phase inoculum (○); logarithmic phase inoculum (●); dashed curves, usual growth curves obtained; lower curve, stationary-phase inoculum; upper curve, logarithmic phase inoculum.

### Table 3. Production of MU9 ds-RNA after infection of Escherichia coli Hfr 3000 cultures grown in corn steep liquor with at least 6 MU9/cell

| Percent corn steep liquor | Flask type | Amount of medium (ml) | Inoculum | 0.05 | 0.1 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 |
|---------------------------|------------|-----------------------|----------|------|-----|-----|-----|-----|-----|-----|
| 5                         | E          | 50                    | S        | 49   | 53  | 74  | 100 | 159 | 64  | 183 |
| 5                         | B          | 20                    | S        | 45   | 80  | 100 | 159 | 116 | 149 | 183 |
| 5                         | B          | 20                    | L        | 63   | 28  | 104 |     |     |     |     |
| 7.5                       | B          | 20                    | L        | 16   | 8   | 20  |     |     |     |     |
| 10                        | B          | 21                    | L        |      |     |     |     |     |     |     |
| 15                        | B          | 20                    | L        |      |     |     |     |     |     |     |

*Abbreviations: E, 250-ml Erlenmeyer flask; B, 250-ml baffled flask; S, inoculated with cells grown to stationary phase in 5% corn steep; L, inoculated with cells growing logarithmically.

*Cells per milliliter at infection (× 10⁻¹⁹).

### DISCUSSION

Infection of nonpermissive cells with MU9 after growth of cultures to high density in corn steep medium has yielded over 300 times the amount of ds-RNA formed by tryptone broth cultures infected with MS2. The MU9 ds-RNA produced in large fermentors is an effective interferon inducer (12).

In all media, cultures infected at approximately one-fourth the final culture density produce the largest quantity of ds-RNA. Although the yield per cell declines from 7 to 8% of the cell dry weight at low density (5 × 10⁸ cells/ml) to 5% at high density (8 × 10⁹ cells/ml), it is more than compensated by the high population present at the time of infection.

The difference in ds-RNA production by cells in low and high-density cultures could be related to a change in either the efficiency of infection or the capacity to synthesize large quantities of RNA. The results available do not exclude either possibility.

If the efficiency of infection were sufficiently reduced at high cell densities, a significant fraction of the population might escape infection. Formation of F pili should be adequate in corn steep medium, because the cells are growing logarithmically at the time of infection (3). Also, accumulation of free F pili released by natural detachment would not interfere with infection (14). Nevertheless, the rate of infection by MU9 of cultures grown to 4 × 10⁹/ml in 5% corn steep liquor medium is slow; sodium citrate added to the culture as late as 10 min after infection reduces ds-RNA production to 10% of the amount formed in its absence.
**TABLE 4. Control of virus infection of cells grown in 5% corn steep liquor**

| Growth medium           | Addition at infection time | MS2* increase (final/initial) | Cellular RNA detected (% of dry weight) | ds-RNA (% of dry weight) |
|-------------------------|----------------------------|-------------------------------|----------------------------------------|--------------------------|
|                         |                            |                               | Corrected                              | Corrected                |
| 5% Corn steep liquor    | None                       | 450                           | 0.3                                    | 1.8                      |
|                         | 0.03 M CaCl₂               |                               | 1.7                                    | 2.7                      |
|                         | 0.06 M CaCl₂               |                               | 0.05                                   | 0.4                      |
| 5% Corn steep liquor +  | None                       | 0.014                         | 0.3                                    | 0.4                      |
| 0.035 M citrate         | 0.01 M CaCl₂               | 500                           | 0.3                                    | 0.4                      |
|                         | 0.03 M CaCl₂               | 560                           | 0.4                                    | 0.7                      |
|                         | 0.06 M CaCl₂               | 340                           | 0.7                                    | 2.1                      |
|                         | 0.01 M MgCl₂               | 420                           | 1.7                                    | 3.0                      |
|                         | 0.03 M MgCl₂               |                               | 2.2                                    |                          |

* Infected with 5 MS2/cell at a density of $4 \times 10^9$ cells/ml.

* Uninfected culture, assayed by standard ds-RNA procedure 3 hr after density reached $4 \times 10^9$/ml.

* Cultures at $4 \times 10^9$/ml infected with MU9. Observed values include ribonuclease-resistant RNA of cellular origin. The resistant RNA present in an equal mass of uninfected cells was subtracted to obtain corrected values.

* Multiplicity of infection.

Slow adsorption could be due to a deficiency of available calcium or magnesium in corn steep liquor medium. Additional calcium increases the amount of viral ds-RNA formed at low multiplicity of infection. Although the elemental analysis of corn steep liquor (courtesy of Anheuser-Busch, Inc.) reveals that these divalent cations are in excess of the amounts present in tryptone medium, a considerable fraction is lost during clarification;
the calcium content of 5% corn steep liquor declined from $1.4 \times 10^{-2}$ m to $5.5 \times 10^{-4}$ m after clarification. The effect of ionic deficiency disappears at higher multiplicities of infection. Infection is adequate in cultures grown to $4 \times 10^9$/ml in 5% corn steep liquor at virus-to-cell ratios greater than 5. A still higher ratio of virus to cells is required to achieve maximum yields when cultures are grown to $8 \times 10^9$/ml in 8.5% clarified corn steep liquor prior to infection. However, only a small portion of the low productivity per cell in high-density cultures can be attributed to infection inadequacy. Addition of divalent metals or increasing the number of infecting virus does not increase the yield per cell to the levels found in low-density cultures.

Although growth rate and ds-RNA per cell are both depressed at very high cell densities, the amount of ds-RNA production is not proportional to growth rate at time of infection. The capacity of cells to produce ds-RNA in corn steep liquor decreases steadily from $5 \times 10^8$ to $8 \times 10^8$ cells/ml, but growth rate does not change significantly until the population exceeds $4 \times 10^9$/ml. Thus, if the efficiency of ds-RNA formation reflects changes in biosynthetic potential during growth in corn steep medium, it is a more sensitive indicator than increase in cell mass.

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