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Asymmetry and Extent of In Vivo Transcripition of R-Plasmid Deoxyribonucleic Acid in *Escherichia coli*

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Deoxyribonucleic acid-ribonucleic acid (DNA-RNA) hybridization studies have been performed with R-plasmid DNA (R538-1*drd*) and in vivo-synthesized RNA. R-plasmid DNA was isolated from *Escherichia coli* K-12, and the complementary strands were separated in cesium chloride-polyuridylic acid-polyguanylic acid gradients. DNA-RNA hybridization was performed with the separated DNA strands and RNA purified from R-plasmid-carrying cells. The results demonstrated that an asymmetric transcription of the R-plasmid DNA occurs in vivo. Hybridization was only detected with the H strand (denser strand in cesium chloride-polyuridylic acid-polyguanylic acid). By determining the density of the RNA-DNA hybrid in CsCl gradients, it was estimated that greater than 60% of the nucleotide sequences in the R-plasmid DNA are transcribed in logarithmically growing *E. coli* cells. No R-plasmid-specific RNA was detected in *E. coli* cells that did not carry the plasmid.

The R-plasmid R538-1*drd* is a self-transmissible plasmid of *Escherichia coli*, which carries drug resistance genes and is transferred at a high frequency from donor to recipient cells (18). The plasmid deoxyribonucleic acid (DNA) molecules are present in about one to two copies per cell and can be isolated as covalently closed circular DNAs of molecular weight 49×10^6 (18).

Although the molecular structure of R-plasmid DNA has been studied in some detail, there have been relatively few studies on the transcription of R-plasmid DNA in *E. coli* (3, 5). Roozen et al. (10) demonstrated that minicells of *E. coli* containing plasmid DNA are able to incorporate [^{3}H]uridine into ribonucleic acid (RNA). The products of the incorporation of [^{3}H]uridine into minicells carrying the bacteriocinogenic plasmid Clo DF 13 (molecular weight 6×10^6) have been characterized by Kool et al. (6). They have demonstrated the appearance of four messenger RNAs specific for the plasmid DNA. Zouzias et al. (21) have studied transcription of the penicillinase plasmid in *Staphylococcus aureus* and have shown that the amount of plasmid-specific messenger RNA is proportional to the amount of plasmid DNA. They have also found that a plasmid mutation that blocked replication of the plasmid led to a decrease in the amount of plasmid messenger RNA.

In this study of the in vivo transcription of R-plasmid DNA in *E. coli*, we prepared RNA from logarithmically growing cells and examined its hybridization to the complementary R538-1*drd* plasmid DNA strands that had been separated by using cesium chloride-polyuridylic acid-polyguanylic acid [CsCl-poly(U,G)] gradients. Hybridization in solution was performed between the separated DNA strands and RNA purified from *E. coli* cells that either did, or did not, carry the R plasmid. The hybrids formed were then analyzed by equilibrium centrifugation in CsCl gradients. Results of these experiments demonstrate that: (i) transcription of the R-plasmid DNA is asymmetric, since RNA hybridization is only detected with the heavy strand isolated from a CsCl-poly(U,G) gradient; (ii) at least 60% of the nucleotide sequences of the R-factor DNA are transcribed in vivo; and (iii) there is little, if any, chromosomally transcribed RNA that is complementary to the R-plasmid DNA.

MATERIALS AND METHODS

Bacterial strains. The R plasmid R538-1*drd* (F-like, carrying resistance to chloramphenicol, streptomycin, and sulfadiazine) was obtained from Elinor Mzynell and transferred by conjugation into *E. coli* K-12 strain AB1702 Thy$^-$ Arg$^-$ (18).

Media and buffers. Bacteria were grown in K+2 medium, which contained M-9 buffer (2), 1% Casamino Acids (Difco), 1% glucose, 10^{-4}% thiamine, and 2 μg of thymidine per ml. TE buffer contained 10 mM 2-amino-2(hydroxymethyl)-1,3-propanediol and 10 mM ethylenediaminetetraacetate (pH 8.0).

Materials. Reagents were obtained from the following sources: pancreatic deoxyribonuclease I, Worthington Biochemicals Corp.; pancreatic ribonuclease (A grade), T1 ribonuclease and lysozyme (A grade), Calbiochem; poly(U,G) (lot no. 2), Miles; [^{3}H]thymidine, New England Nuclear. All other chemicals were of reagent grade.

Preparation of DNA. [^{3}H]thymidine-labeled R-plasmid DNA was purified as described previously (20). The complementary strands were separated by equilibrium centrifugation in a CsCl-poly(U,G) gradient, treated with 0.3 N KOH (18 h at 37 C) to remove the poly(U,G), and dialyzed against 0.3 M NaCl-TE buffer (19). Final DNA concentration was adjusted to 1 μg/ml based on a calculated specific activity of 4×10^4 counts/min per μg.

Preparation of RNA. RNA was purified by a modification of the sodium dodecyl sulfate precipitation method of Summers (14). Logarithmically growing cells (5×10^8/ml) were treated with NaN_3 (20 mM final concentration) and immediately poured onto crushed ice. Cells were collected by centrifugation at 0 C, suspended in 10% sucrose, and converted to spheroplasts with lysozyme-ethylenediaminetetraacetic acid (20). After 10 min at 0 C the spheroplasts were lysed by addition of sodium dodecyl sulfate (1% final concentration) and incubated at 37 C for 10 min. NaCl was then added to a final concentration of 1 M. After standing overnight at 0 C, the precipitate, containing the bulk of the chromosomal DNA, was removed by centrifugation at 0 C (20 min at 27,000 $\times$ *g*) and discarded. Two volumes of 100% ethanol were added to the supernatant. After several hours at -20 C, the precipitate was collected by centrifugation (5 min at 3,000 $\times$ *g*), suspended in TE buffer containing 0.05 M NaCl, and dialyzed overnight against the same buffer. Pancreatic deoxyribonuclease I and $MgSO_4$ were added to final concentrations of 20 μg/ml and 20 mM, respectively. The mixture was incubated for 1 h at 37 C to degrade the remaining DNA, and extracted two times with phenol saturated with 0.1 M tris(hydroxymethyl)aminomethane (pH 8.0). The aqueous layer containing the RNA was then precipitated with 2 volumes of ethanol at -20 C, centrifuged, suspended in TE buffer, and dialyzed against several changes of TE buffer containing 0.3 M NaCl. RNA concentration was determined spectrophotometrically by its absorbance at 260 nm and adjusted to 10 mg/ml with TE buffer containing 0.3 M NaCl.

RNA-DNA hybridization. Samples of RNA and DNA (final volume, 0.2 ml; final concentration of RNA and DNA, 10 mg and 1 μg per ml, respectively) were mixed and incubated at 68 C in Beckman microfuge tubes. At the end of the hybridization (usually 20 h), the samples were treated with pancreatic ribonuclease (20 μg/ml final concentration) and T1 ribonuclease (10 U/ml) for 1 h at 23 C as described by Bøvre and Szybalski (2). The pancreatic ribonuclease and T1 ribonuclease were boiled for 5 min before use to inactivate any contaminating deoxyribonuclease. Annealed samples were added to 3.3 ml of CsCl, and the density was adjusted to 1.7 g/cm^3. Double-stranded phage T7 DNA, labeled with [^{14}C]thymine, was added as a density marker, and the samples were centrifuged for 60 h at 33,000 rpm at 20 C in a Spinco SW50.1 rotor. Gradients were fractionated as previously described (19), and samples were counted for radioactivities in a Beckman scintillation counter.

RESULTS

The separation of the complementary strands of the R538-1*drd* plasmid DNA in a CsCl-poly(U,G) gradient is shown in Fig. 1. The purity of these preparations was determined by annealing DNA from each of the two peaks separately and by annealing an equimolar mixture of DNA from both peaks. Since duplex DNA is formed only between the complementary strands, the distribution of single- and double-stranded DNA after annealing is an index of strand purity. The relative amounts of single- and double-stranded DNA were determined by chromatography and hydroxyapatite (Fig. 2; 9). By the above criteria, the two peaks were found to contain between 80 and 90% pure complementary strands.

The extent and specificity of in vivo transcription of the R538-1*drd* plasmid was determined by hybridizing RNA isolated from R-plasmid-carrying strains with ^{3}H-labeled, separated strands of the R-plasmid DNA. By using the separated strands, DNA-DNA hybrid formation was avoided. We chose to do the hybrid-

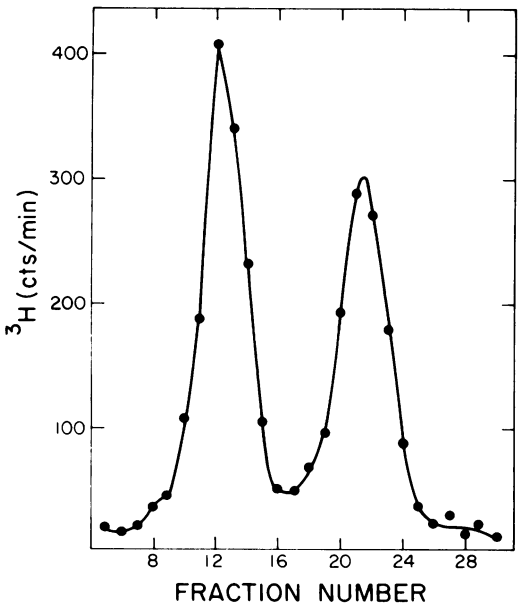

FIG. 1. *Equilibrium centrifugation CsCl-poly(U,G) of unit-length R538-1drd plasmid DNA strands. Centrifugation was for 48 h at 35,000 rpm at 20 C in a Spinco 50Ti rotor.*

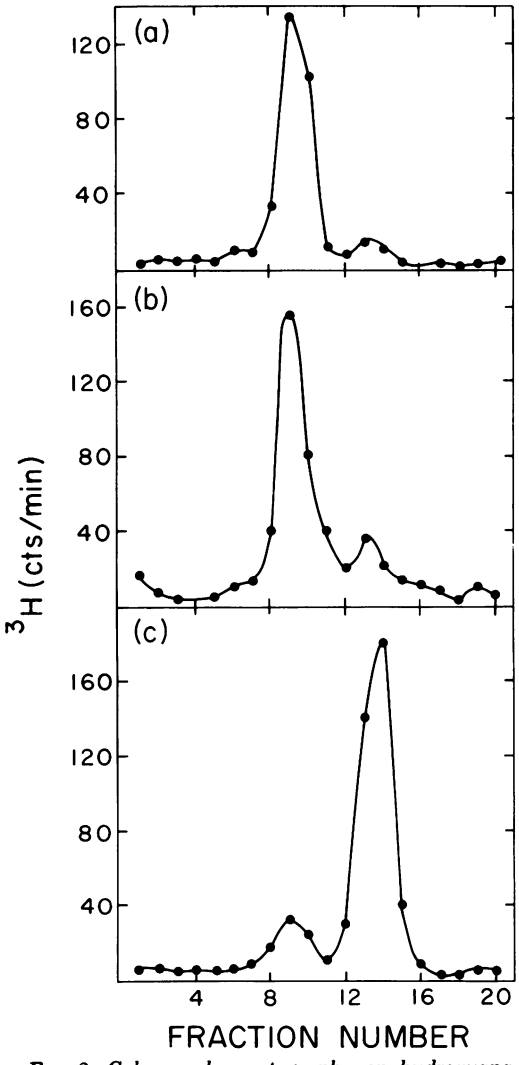

FIG. 2. *Column chromatography on hydroxyapatite of DNA isolated from CsCl-poly(U,G) and annealed. DNAs from each of the two peaks, as well as an equimolar mixture of DNA from both peaks, were annealed at 68 C for 20 h. The DNA was applied to a hydroxyapatite column and eluted with a linear 0.05 to 0.35 M sodium phosphate gradient. Single-stranded DNA eluted at fractions 8 through 10 and double-stranded DNA eluted at fractions 13 through 15. (a) Light peak from CsCl-poly(U,G); (b) heavy peak from CsCl-poly(U,G); (c) equimolar mixture of heavy and light peaks.*

ization in liquid using an excess of nonradioactive RNA because of the difficulty in isolating large amounts of R-plasmid DNA and so that the hybrids could be characterized further. Figures 3a and b show the radioactivity profile obtained from a CsCl gradient after 0 and 20 h of hybridization with the H and L strands of the R-plasmid DNA and RNA isolated from the R538-1*drd* plasmid-carrying strain. At time zero (Fig. 3a), both the H and L strands banded at a density of 1.725 g/cm^3, the density of the unhybridized strands. After 20 h of hybridization (Fig. 3b), the H strand shifted to a density of 1.783 g/cm^3, indicating that extensive RNA hybridization occurred with the H strand. The L strand, however, still banded at a density of 1.725 g/cm^3, indicating that little hybridization of the RNA occurred even after 20 h of incubation at 68 C. These results show that there is extensive transcription of the H strand in vivo, and little or no transcription of the L strand.

Control experiments (data not shown) demonstrated that hybridization had gone to com-

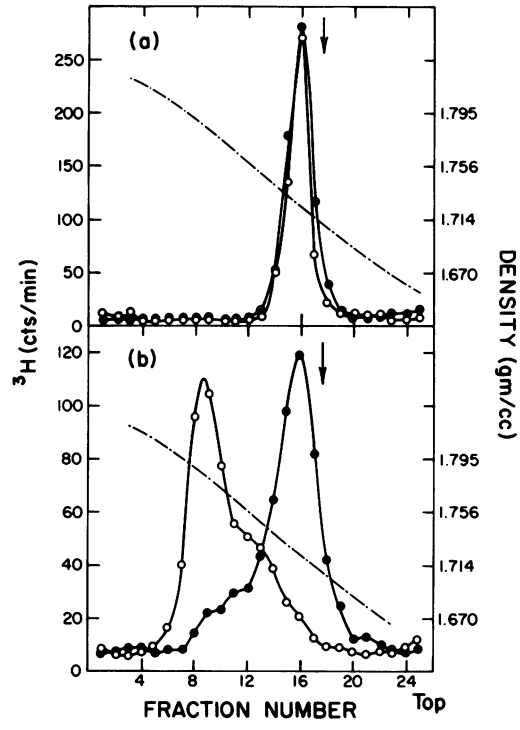

FIG. 3. *Equilibrium centrifugation in CsCl after hybridization of ^{3}H-labeled H- or L-strand DNA and unlabeled RNA prepared from a R38-1drd plasmid-carrying strain of E. coli. Hybridization was performed as described in Materials and Methods. The arrow indicates the position of ^{14}C-labeled double-stranded T7 DNA added as a density marker. Densitites were determined by measuring the refractive index of individual fractions. The results from two different gradients (one for the H strand and one for L strand) have been combined in the plots of (a) and (b). Centrifugation was for 60 h at 33,000 rpm at 20 C in a Spinco SW50.1 rotor. (a) 0 h of hybridization; (b) 20 h of hybridization. Symbols: O, H strand; ●, L strand; (— · — · —) density.*

pletion in these experiments, since longer periods of incubation or increasing the RNA concentration up to threefold did not result in a further shift in density of the H strand.

Based on the measured density of the hybrid in CsCl, the fraction of the H strand transcribed can be calculated using the relationship: percent DNA hybridized = [(measured ρ of hybrid $-$ ρ of H strand)/(hypothetical ρ of 1:1 RNA-DNA hybrid $-$ ρ of H strand)] $\times$ 100. Using a density of 1.710 g/cm^3 for the T7 marker DNA (16), the density of strand H is calculated to be 1.725 g/cm^3. This is the same as the density of ϕX-174 DNA (1.725). Thus, the density of a 1:1 RNA-DNA hybrid between the H strand and complementary RNA would be expected to have a density of 1.815 g/cm^3, similar to that of a 1:1 ϕX-174 DNA-RNA hybrid (12). The measured density of the hybrid formed between the H strand and complementary RNA in CsCl was 1.783 g/cm^3. Substituting these values in the above equation, we calculate that at least 64% of the nucleotide sequences of the R538-1*drd* plasmid was hybridized to RNA and was thus transcribed in logarithmically growing *E. coli* cells that carried the plasmid.

The validity of the above analysis was tested experimentally by performing hybridization experiments with the separated strands of T7 DNA and RNA isolated from T7-infected cells at 10 min postinfection. At this time the level of hybridization is at a maximum (15). Hybrids were analyzed as described in Fig. 3. From the position of the hybrid in CsCl, we calculated that 75% of the *r* strand of T7 was being transcribed. Based on the size of the T7 mRNAs, Summers (13) estimated that 90 to 100% of the *r* strand was being transcribed in infected cells. This discrepancy could indicate that the method of CsCl analysis of hybrids results in an underestimation of the level of hybridization.

The specificity of the hybridization shown in Fig. 3 was tested further by performing hybridizations between the H strand of the R plasmid and RNA isolated from *E. coli* cells that did not carry the R plasmid (Fig. 4). (Conditions of hybridization were the same as those of Fig. 3.) No detectable density shift was observed after 20 h of hybridization, indicating that there is little, if any, chromosomally transcribed RNA that is complementary to the R-plasmid DNA.

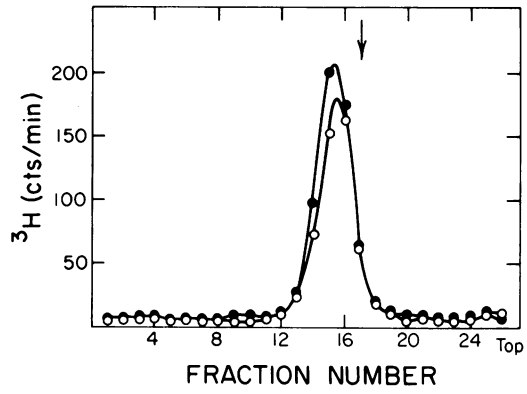

FIG. 4. *Equilibrium centrifugation in CsCl after hybridization between ^{3}H-labeled H-strand DNA and unlabeled RNA prepared from an isogenic strain of E. coli that did not carry the R538-1drd plasmid. Hybridization was performed as described in Materials and Methods. The arrow indicates the position of ^{14}C-labeled double-stranded T7 DNA added as a density marker. Symbols: ●, 0 h of hybridization; ○, 20 h of hybridization.*

DISCUSSION

The results of these experiments demonstrate that an asymmetric transcription of the R538-1*drd* plasmid DNA occurs in vivo. This is based on our finding that plasmid-specific RNA hybridizes with only one of the complementary strands of the R538-1*drd* DNA, the denser strand isolated from a CsCl poly(U,G) gradient. Based on the measured density of the hybrid in CsCl equilibrium gradients, we calculated that at least 60% of the nucleotide sequences of the R538-1*drd* plasmid are transcribed in vivo. RNA isolated from cells that did not carry the R plasmid did not show a detectable level of hybridization to R-plasmid DNA. This result demonstrates that little, if any, RNA homologous to the R plasmid is present in cells that do not carry the plasmid. Although the degree of homology between the R-plasmid DNA and the *E. coli* chromosome has not been determined, Falkow and Citarella (4) have shown by DNA-DNA hybridization that the F plasmid contains approximately 40% homology with the *E. coli* chromosome. This relatively high level of homology is presumably involved in the formation of stable Hfr cells and F′ factors. Since R plasmids rarely appear to stably integrate into the chromosome, it might be expected that they would have less homology with the chromosome than F factors (8). Our results suggest that little homology exists between the R538-1*drd* plasmid and transcribed regions of the host chromosome, and that any homology that might exist would be limited to nontranscribed regions of the chromosome.

Examining transcription patterns of the penicillinase plasmid from *S. aureus*, Zouzias et al. (21) found that there was about 6% as much

hybridization, to the plasmid, of pulse-labeled RNA isolated from a plasmid-minus strain as there was from a plasmid-containing strain. However, DNA-DNA hybridization experiments demonstrated that only about 1% of the nucleotide sequences of the plasmid were complementary to the chromosome (21). In the present experiments, a similar low level of complementarity between plasmid DNA and chromosomal DNA would not result in a detectable density shift in CsCl even if the 1% homology region was completely transcribed. Although our experiments demonstrate that at least 60% of the nucleotide sequences of the R-plasmid DNA are being transcribed, it remains to be determined whether these transcripts are limited to one large and contiguous region, or are from several regions on the DNA. M. Achtman (personal communication) has recently suggested that the transfer genes carried by the F factor are part of an operon that could produce a large polycistronic mRNA. We are currently attempting to visualize the DNA-RNA hybrids in the electron microscope to determine whether transcription is limited to several areas of the R plasmid.

The R plasmid R538-1*drd* used in these studies is derepressed for its ability to transfer between R^+ and R^- cells, suggesting that the genes concerned with pilus formation and transfer are being continuously expressed. Consistent with this view, preliminary experiments comparing the level of transcription of R-plasmid genes in wild-type, repressed R-plasmid R538-1 with derepressed R538-1*drd* have shown reduced transcription in the strain that carries the repressed R plasmid.

Asymmetric transcription of several phage DNAs has been found in *E. coli* (phages T7 and T3 [15] and ϕII [7]), *Bacillus subtilis* (phage alpha [20]), and *B. stearothermophilus* (phage TP-84 [11]). Although the reason for this asymmetry of transcription is not clear, it obviously plays an important role in regulation, as well as restricting the events involved in the evolution of these DNA molecules. Though the reason for the asymmetric transcription of the R-plasmid DNA is not known, it may be significant that the transcribed strand is the same one which is transferred from donor to recipient cells during conjugation (18).

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages, p. 446. Wiley-Interscience, New York.
2. Bøvre, K., and W. Szybalski. 1971. Multistep DNA-RNA hybridization techniques, p. 350. *In* Methods in enzymology, vol. 21. Academic Press Inc., New York.
3. Clowes, R. C. 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. **36**:361-405.
4. Falkow, S., and R. V. Citarella. 1965. Molecular homology of F-merogenote DNA. J. Mol. Biol. **12**:138-151.
5. Helinski, D. R. 1973. Plasmid determined resistance to antibiotics: molecular properties of R factors. Annu. Rev. Microbiol. **27**:437-470.
6. Kool, A. J., M. S. van Zeben, and H. J. J. Nijkamp. 1974. Identification of messenger ribonucleic acids and proteins synthesized by the bacteriocinogenic factor CloDF13 in purified minicells of *Escherichia coli*. J. Bacteriol. **118**:213-224.
7. Linial, M., M. H. Malamy, and W. C. Summers. 1970. Separation of the complementary strands and asymmetric transcription of coliphage ϕII DNA. Virology **41**:766-767.
8. Meynell, E., and N. Datta. 1969. Sex-factor activity of drug-resistance factors, p. 120-133. *In* Ciba Foundation symposium on bacterial episomes and plasmids. Churchill, London.
9. Miyazawa, Y., and C. A. Thomas, Jr. 1965. Nucleotide composition of short segments of DNA molecules. J. Mol. Biol. **11**:223-237.
10. Roozen, K. J., R. G. Fenwick, Jr., and R. Curtiss III. 1971. Synthesis of ribonucleic acid and protein in plasmid-containing minicells of *Escherichia coli* K-12. J. Bacteriol. **107**:21-33.
11. Saunders, G. F., W. N. Reese, J. Bergendahl, and M. Mandel. 1969. The binding of polyribonucleotides to single-stranded TP-84 deoxyribonucleic acid. Biochemistry **8**:1222-1227.
12. Sinsheimer, R. L., and M. Lawrence. 1964. *In vitro* synthesis and properties of ϕX DNA-RNA hybrids. J. Mol. Biol. **8**:289-296.
13. Summers, W. C. 1969. Characterization of T7 RNA by polyacrylamide electrophoresis analysis. Virology **39**:175-182.
14. Summers, W. C. 1970. A simple method for extraction of RNA from *E. coli* utilizing diethyl pyrocarbonate. Anal. Biochem. **33**:459-463.
15. Summers, W. C., and W. Szybaski. 1968. Totally asymmetric transcription of coliphage T7 *in vivo*: correlation with poly G binding sites. Virology **34**:9-16.
16. Szybalski, W. 1968. Use of cesium sulfate for equilibrium density gradient centrifugation, p. 330. *In* Methods in enzymology, vol 12B. Academic Press Inc., New York.
17. Tocchini-Valentini, X., M. Stodolsky, A. Aurisicchio, M. Sarnat, F. Graziosi, S. B. Weiss, and E. P. Geiduschek. 1963. On the asymmetry of RNA synthesis *in vivo*. Proc. Nat. Acad. Sci. U.S.A. **50**:935-942.
18. Vapnek, D., M. B. Lipman, and W. D. Rupp. 1971. Physical properties and mechanism of transfer of R factors in *Escherichia coli*. J. Bacteriol. **108**:508-514.
19. Vapnek, D., and W. D. Rupp. 1970. Asymmetric segregation of the complementary sex-factor DNA strands during conjugation in *Escherichia coli*. J. Mol. Biol. **53**:287-303.
20. Vapnek, D., and W. D. Rupp. 1971. Identification of individual sex-factor DNA strands and their replication during conjugation in thermosensitive DNA mutants of *Escherichia coli*. J. Mol. Biol. **60**:413-424.
21. Zouzias, D., M. Rush, E. Murphy, and R. Novick. 1973. Studies on plasmid replication. II. *In vivo* transcription and its control in penicillinase plasmids from *Staphylococcus aureus*. J. Mol. Biol. **79**:471-480.