Volume 7 Number 3 1979 Nucleic Acids Research

Gene expression in vitro of colicin E1 plasmid

Yousuke Ebina, Fumio Kishi, Teruko Nakazawa and Atsushi Nakazawa

Department of Biochemistry, Yamaguchi University School of Medicine, Kogushi, Ube, Yamaguchi, 755 Japan

Received 19 July 1979

ABSTRACT

Among eighteen polypeptides synthesized *in vitro* from colicin E1 plasmid, one of the major products with a molecular weight of 59,000 was identified as colicin E1 by its immunological property, molecular size, and biological activity. In addition to this polypeptide, seven other polypeptides reacted with colicin E1 antiserum. Using *Eco*RI-cleaved colicin E1 DNA, a 56,000-dalton polypeptide of truncated colicin E1 was synthesized, but no polypeptide that reacted with colicin E1 antiserum was produced from *Sma*I-cleaved colicin E1 DNA. This fact indicates that the direction of transcription of colicin E1 structural gene is from *Sma*I site to *Eco*RI site *in vitro*. The immunity protein of a molecular weight of 14,300 and a component of relaxation proteins of a molecular weight of 64,000 were deduced by comparing the results of the gene expression *in vitro* of one-half (pAO100) and a quarter (pAO2) of colicin E1 plasmid. The directions of transcription-translation in the genes on the plasmid were discussed. The colicin E1 plasmid appears to have at least three transcriptional units.

INTRODUCTION

Colicin E1 plasmid (ColE1) has been extensively used as a model replicon for studies on DNA replication (1-3) and as a vector for cloning of DNA fragments (4). However, little is known about its gene expression. The plasmid ColE1 is normally dormant with regard to active colicin production, but it is readily induced to synthesize colicin E1 by agents such as mitomycin C and ultraviolet light. The induction occurs as the result of derepression of the colicin E1 gene and the process is thought as a part of the SOS function which is dependent on the functional gene of *recA* or *lexA* (5-7).

In order to clarify the regulation of the gene expression, it is necessary to analyze gene products of the plasmid ColE1. The analysis of the polypeptides synthesized by ColE1 has been examined in minicells containing ColE1 derivatives which were formed by deletions or insertions of transposons (8-10). On the other hand, Eichenlaub reported the synthesis of active

colicin El in the cell-free system (11). We analyzed polypeptide products labeled with ^{14}C-amino acids in the cell-free system. A similar approach was taken by Yang and Zubay (12) and Collins *et al.* (13).

In this paper, some of the gene products such as colicin El protein, immunity protein, and a relaxation protein were assigned using native ColEl, restriction endonuclease-cleaved ColEl DNAs, and one-half and a quarter plasmids of ColEl as templates. The directions of transcription-translation in these genes were discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids —— Strains W3110 and Y20(ColEl) have been described (14). Strain C600 containing one-half of ColEl plasmid, pAO100 or a quarter plasmid, pAO2 (15) was generously provided by Dr. A. Oka. All strains are derivatives of *Escherichia coli* K-12.

Preparations of plasmid and T4 DNA —— Y20(ColEl) was grown at 37°C in the minimal salt medium (M-9) supplemented with vitamin-free casamino acids (0.15%), thiamine-HCl (10 µg/ml) and glucose (0.4%) (14). Chloramphenicol (150 µg/ml) was added at a cell density of 3 X 10^8/ml. After 15-h incubation, the cells were harvested by centrifugation. The preparation of cleared lysate and purification of ColEl by CsCl-ethidium bromide equilibrium centrifugation were performed as described by Clewell and Helinski (16). The stock solution of ColEl used in this study contained about 60% closed circular and 40% open circular molecules as judged by agarose gel electrophoresis. pAO2 and pAO100 DNAs were prepared by the same method as described above except that the medium used for growth was E-broth (15). T4 DNA was obtained by phenol extraction of the phage particles (17). The concentration of DNA was determined fluorometrically with diaminobenzoic acid (18).

Cleavage of ColEl DNA with *Eco*RI and *Sma*I endonuclease —— The reaction mixture for *Eco*RI cleavage contained 50 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 50 mM NaCl, 135 µg ColEl DNA and 300 units of *Eco*RI (Boehringer Mannheim) in 500 µl. After incubation at 37°C for 4 h, the reaction mixture was dialyzed overnight against 1 liter of 0.01 M Tris-HCl (pH 7.5)-1 mM EDTA. The reaction mixture for *Sma*I cleavage contained 15 mM Tris-HCl (pH 8.5), 6 mM $MgCl_2$, 15 mM KCl, 67.5 µg ColEl DNA and 300 units of *Sma*I (Boehringer Mannheim) in 200 µl. The reaction mixture was incubated at 25°C for 5 h and dialyzed overnight against 1 liter of 0.01 M Tris-HCl (pH 7.5)-1 mM EDTA. Under these conditions, ColEl DNA was completely cleaved as judged by agarose gel elec-

trophoresis and electron microscopy.

Protein synthesis *in vitro* —— Cell-free protein synthesis was performed as described by Zubay *et al.*(19) except that the strain used here for preparation of S-30 was *E. coli* W3110. The standard reaction mixture for protein synthesis contained 2.7 μg ColE1 DNA, 1.3 mg protein of S-30, 2.8 mM dithiothreitol, 1.1 mM each of CTP, GTP, UTP, and 4.4 mM ATP in 50 μl. After incubation of the reaction mixture at 37°C for 70 min, the colicin activity was assayed as described previously (14).

Fluorography of sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis —— For the detection of polypeptide products, incubation was carried out either with 4 μCi of L-[U-^{14}C]leucine (specific activity, 354 mCi/nmol, Radiochemical Centre) instead of cold leucine in the reaction mixture described above or with 4 μCi of [U-^{14}C]protein hydrolysate (specific activity, 59 mCi/milliatom carbon, Radiochemical Centre) to the above mixture. After incubation, the mixture was washed with 5 ml of 10% trichloroacetic acid containing 4.4 mM each of 20 amino acids, and trichloroacetic acid was removed by extraction with water-saturated ethylether. The proteins were treated as described by Korn and Wright (20) and used as samples for SDS-polyacrylamide gel electrophoresis according to Laemmli (21). Electrophoresis was carried out on the slab of 10, 12 or 15% polyacrylamide gel containing SDS (13.5 X 10 X 0.1 cm) with a constant current of 50 mA. The slab was subjected to fluorography by the method of Bonner and Laskey (22). The film was exposed to the gel at -70°C for 24 h. Molecular weight standards of protein were bovine serum albumin (68,000), hen egg albumin (45,000), chymotrypsinogen (25,000) and lysozyme (14,300).

Immunoprecipitation of ^{14}C-labeled protein synthesized *in vitro* —— A rabbit was injected subcutaneously three times with 50 μg of purified colicin E1 protein (23) emulsified in Freund's complete adjuvant (Iatron Laboratories Co., Tokyo) over a 2-week period. The animal was bled 1 week after the last injection to obtain the antiserum. The titer of the antibody was estimated by Ouchterlony double-diffusion method (24). To purify γ-globulin fraction, a half volume of the saturated ammonium sulfate solution (pH 6.9) was added to the antiserum and the mixture was centrifuged. The precipitate was dissolved in saline and dialyzed against 3 liters of 0.02 M borate buffer (pH 8.0)-0.15 M NaCl for 3 days. The γ-globulin fraction was stored at -20°C. A modification of the method of Kessler (25) was used to precipitate the antigen-antibody complex with the strain Cowan I of *Staphylococcus aureus* (protein A producer). Cells of the strain, which was generously provided by Dr.

T. Tada (Univ. of Tokyo), were fixed with formaldehyde and killed by heat (25). Fifty µl of the reaction mixture after protein synthesis *in vitro* were incubated with 5 µl (9 µg) of the partially purified γ-globulin and 45 µl of saline at 4°C for 15 min. To the above mixture were added 10 µg of the *S. aureus* cells in 100 µl of 0.5% NP40 (Nakarai Chemical Co., Kyoto, Japan), 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% sodium azide, and 1 mg/ml bovine serum albumin (pH 7.4) (detergent buffer solution). After incubation for 10 min at 4°C, 0.5 ml of the detergent buffer solution was added and the mixture was centrifuged to collect the bound antigen-antibody complex. After washing the precipitate 3 times with 0.5 ml of the detergent buffer solution, the antigen and antibody were eluted from the cell pellet by incubating with 80 µl of 2% SDS, 40 mM dithiothreitol, 10% sucrose, 10 mM Tris-HCl (pH 8.0) at 100°C for 5 min. This eluate was subjected to SDS-polyacrylamide gel electrophoresis as described above.

RESULTS AND DISCUSSION

Colicin El activity was detected on incubation for protein synthesis *in vitro* with ColEl DNA. The activity increased proportionally with concentrations of ColEl DNA added up to 54 µg/ml. No colicin activity was found with T4 DNA or in the absence of ColEl DNA. On analysis of radioactive proteins synthesized with ^{14}C-amino acid on 10% SDS-polyacrylamide gel electrophoresis followed by fluorography (Fig. 1), a number of discrete bands were seen in the presence of ColEl DNA, while in the absence of ColEl DNA few protein bands could be seen. A protein band with a molecular weight of 59,000, whose intensity increased with concentrations of ColEl DNA, was identified colicin El protein by comigration with purified colicin El in SDS-gel electrophoresis and by immunoprecipitation by a specific antiserum for colicin El (Fig. 2-a').

At least 13 polypeptide-bands of molecular weights larger than 15,000 could be distinguished in the fluorography (Fig. 2-a). Five additional bands of molecular weights below 15,000 could be separated in a 15% acrylamide gel (result not shown). In addition to 59,000-dalton colicin El protein, polypeptides of 51,000, 46,000, 39,500, 36,000, 33,000, 30,000 and 25,000 daltons also reacted with colicin El antiserum (Fig. 1-a'). The immunoprecipitated polypeptides other than 59,000-dalton polypeptide were degradation products or products by premature termination of colicin El synthesis *in vitro* as in the case of β-galactosidase synthesis *in vitro* (26). The distinct band of 46,000 daltons in immunoprecititated polypeptides (Fig. 2-a',b') was broad

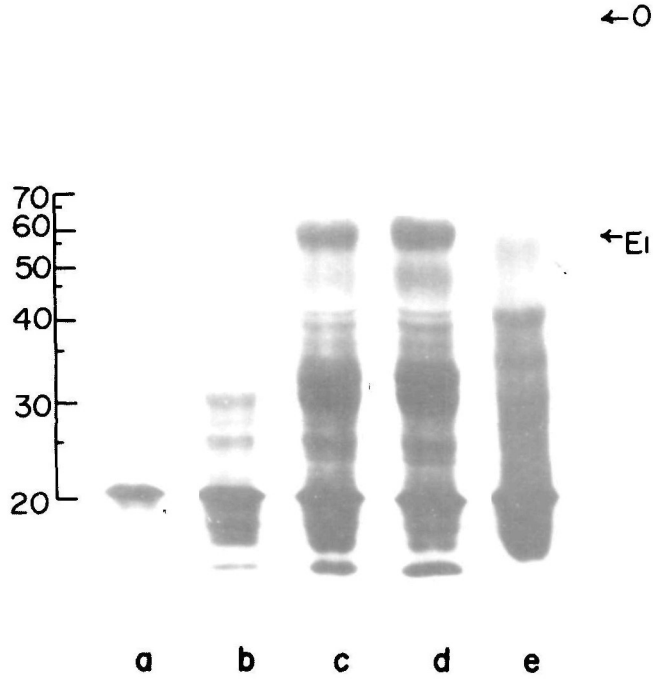

Fig. 1 SDS-polyacrylamide gel electrophoresis after *in vitro* protein synthesis with [^{14}C]leucine. Following concentrations of ColE1 DNA were used: (a) 0 µg/ml, (b) 18 µg/ml, (c) 36 µg/ml, (d) 54 µg/ml. Slot (e) is the result with 54 µg/ml of T4 DNA. Electrophoresis was carried out in 10% SDS-polyacrylamide slab gel. Scale of molecular weights (X 10^{-3}) determined by standard proteins, the position of colicin E1 (E1) and the origin of electrophoresis (O) were indicated.

in the untreated sample (Fig. 2-a). The band, however, is unlikely to be due to degradation of colicin E1 during immunoprecipitation, since the addition of a protease inhibitor, diisopropyl phosphofluoridate or phenylmethyl-

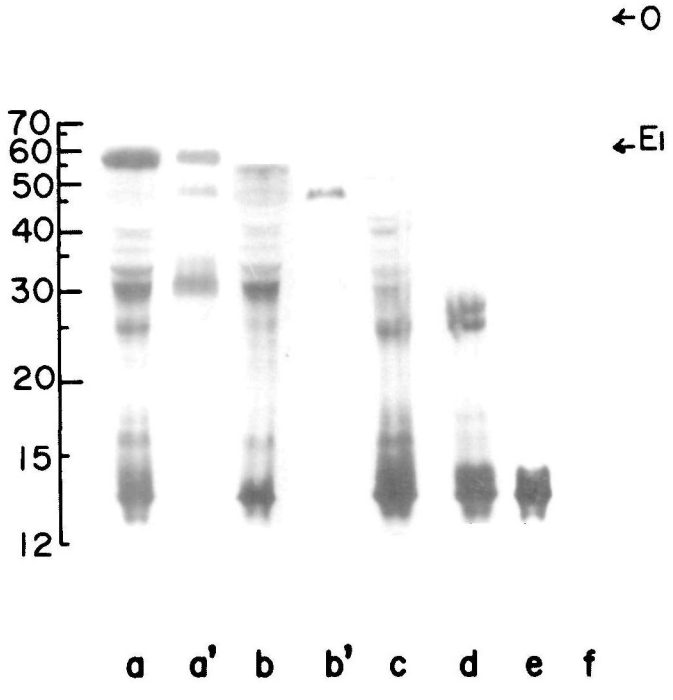

Fig. 2 SDS-polyacrylamide gel electrophoresis after *in vitro* protein synthesis with [^{14}C]protein hydrolysate. (a) ColE1 DNA (54 µg/ml), (a') immunoprecipitated polypeptides from incubation mixture in (a), (b) *Eco*RI-cleaved ColE1 DNA (64 µg/ml), (b') immunoprecipitated polypeptides from incubation mixture in (b), (c) *Sma*I-cleaved ColE1 DNA (64 µg/ml), (d) pAO100 DNA (116 µg/ml), (e) pAO2 DNA (116 µg/ml), (f) no DNA. For the experiments in slot b, b', and c, 8 µCi of [^{14}C]protein hydrolysate were used. The samples were subjected to electrophoresis in 12% SDS-polyacrylamide slab gel. Scale of molecular weight (X 10^{-3}), the position of colicin E1 (El) and the origin of electrophoresis (O) were indicated. S-30 used in this experiment was of a batch different from that used in Fig. 1.

sulfonylfluoride to the reaction mixture for the immunoprecipitation gave similar results.

The 59,000-dalton polypeptide was not synthesized by *Eco*RI-cleaved ColE1 DNA, but a 56,000-dalton polypeptide which reacted with colicin E1 antiserum was synthesized (Fig. 2-b,b'). The structural gene for colicin E1 lies between 0.22-0.23 and 0.99 map units across the *Eco*RI site according to the experiments using transposons (27) and the sequence data of pAO2 (28). The size of the peptide (95% of colicin E1) corresponds with that of the colicin E1 structural gene cleaved with *Eco*RI (Fig. 3). The *Sma*I-cleaved ColE1 DNA could produce neither the 59,000-dalton polypeptide (Fig. 2-c), nor the other polypeptides reacted with colicin E1 antiserum (data not shown). Thus, the direction of transcription of colicin E1 structural gene appears from *Sma*I site to *Eco*RI site as reported previously (9, 12, 27). In order to get the same level of isotope incorporation into the proteins other than colicin E1, at least twice as much radioisotope had to be used with the linear form as with the closed and open circular forms (see legend to Fig. 2). Thus, the

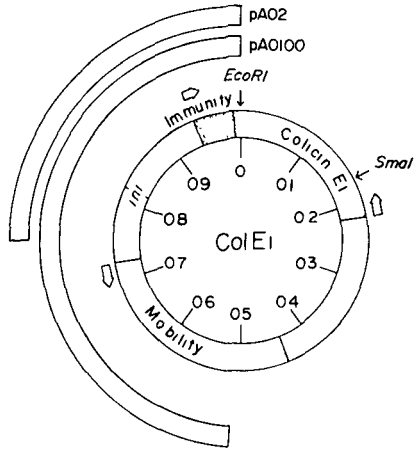

Fig. 3 Map of ColE1 plasmid. Map unit is the fractional length from the 5' end of the H-strand generated by cleavage of ColE1 DNA by EcoRI, and therefore the *Eco*RI site is at 0.00 (1.00) unit. ColE1 DNA replication initiates at *ini* (0.82 unit). The gene loci required for expression of immunity (0.94-0.99 units), colicin E1 (0.22-0.99 units) and plasmid mobility (0.44-0.72 units) were determined by the sequence analysis of pAO2 (28), and by the experiments of ColE1 derivatives containing deletions or insertions of transposons (27). The pAO2 plasmid has the region of 0.75 to 1.00 units (15). The pAO100 has the region of 0.51 to 1.00 units and a 300-nucleotide segment of ϕ80ptl90 DNA (31). Presumable directions of transcription of each gene are indicated by arrows.

efficiency of transcription-translation by the linear form of ColE1 DNA which was cleaved by restriction enzymes was less than that by the closed and open circular forms used as a template. A similar conclusion was reached by Levine and Rupp (29) and Collins *et al.* (13). The colicin E1 activity was scarcely detected after incubation for protein synthesis *in vitro* with *Eco*RI-cleaved ColE1 DNA. Taking into account of the amount of the truncated colicin E1 protein synthesized, the specific colicin activity of the incomplete polypeptide of colicin E1 is less than 1%. The result can be understood by the finding that the C-terminal portion of colicin E1 is essential for the colicin activity (30).

The plasmid pAO2 (0.75-1.00 map units, Fig. 3) (15) synthesized following four polypeptides; 14,300, 13,800, 13,200 and 12,500 daltons (Fig. 2-e). According to Oka *et al.* (28), products of *in vitro* transcription from pAO2 DNA using purified RNA polymerase were RNAs of 360 and 110 nucleotides. A similar result was obtained by Levine and Rupp (29). The former RNA could code for a polypeptide of 113 amino acid residues, and appeared to be derived from the gene for colicin immunity. The latter RNA of 110 nucleotides could not be translated, because there is no initiation codon in the nucleotide sequence of this RNA (28). We presume that the 14,300-dalton polypeptide appeared in our experiments is the immunity protein and 13,800, 13,200, 12,800-dalton polypeptides are premature termination or degradation products of the immunity protein. The molecular weight of immunity protein well corresponds to that found in minicells (9, 10).

The plasmid pAO100 having a molecular weight of 2.1×10^6 consists of approximately one-half of the ColE1 genome (0.51-1.00 map units) and an about 300-nucleotide segment of ϕ80pt190 DNA as shown in Fig. 3 (31). Among nine polypeptides synthesized *in vitro* from pAO100 (Fig. 2-d), the 54,000-dalton polypeptide is presumably derived from the relaxation protein. This peptide was not synthesized from pAO2 and also was not detected in the bands directed by ColE1 DNA and ColE1 DNAs cleaved by restriction enzymes. Two polypeptides larger than this polypeptide are directed by the whole genome of ColE1 plasmid, one of which is identified as colicin E1. The other peptide of 64,000 daltons was faint, but significantly was found in protein bands from either *Eco*RI-cleaved or *Sma*I-cleaved ColE1 DNA, which is almost the same size as a component of relaxation proteins reported by Lovett and Helinski (32). This polypeptide could be hardly detected in ColE1-directed bands because of the presence of a large amount of colicin E1 just below this peptide. The map position of the gene participating in the mobilization of ColE1 DNA

TABLE 1. Summary of molecular weights of polypeptides synthesized by ColE1 DNA and its derivatives *in vitro*

Molecular weight X 10^{-3}					Presumptive function
ColE1	*Eco*RI[a]	*Sma*I[b]	pAO100	pAO2	
(64)	64	64			Mobility
59*					Colicin
	56*				Colicin related
			54		Mobility related
51*	51*				Colicin related
46*	46*				Colicin related
44	44	44			Unknown
41	41	41			Unknown
39.5*	39.5*				Colicin related
36*	36*	36			Colicin related and unknown
33*	33*	33			Colicin related and unknown
30*	30*	30			Colicin related and unknown
			27		Mobility related
25*	25	25	25		Colicin and mobility related
17	17	17	17		Mobility related
15.5	15.5	15.5	15.5		Mobility related
15	15				Unknown
14.3	14.3	14.3	14.3	14.3	Immunity
13.8	13.8	13.8	13.8	13.8	Immunity related
13.2	13.2	13.2	13.2	13.2	Immunity related
12.8	12.8	12.8	12.8	12.8	Immunity related
12		12			Unknown

*Polypeptides reacted with colicin E1 antiserum. [a]*Eco*RI-cleaved ColE1 DNA. [b]*Sma*I-cleaved ColE1 DNA.

is between 0.44 and 0.72 map units (Fig. 3) (27). The size of the polypeptide capable of coding for by this region of ColE1 DNA is about 68,000 daltons. Since the relaxation protein is considered to play a role in the plasmid mobilization and they consist of three proteins of 60,000, 16,000, and 11,000 daltons (27, 32), the 64,000-dalton peptide synthesized *in vitro* could be ascribed to the largest component of relaxation proteins. The 62,000-dalton polypeptide which corresponds to a largest component of relaxation proteins was reported to be synthesized by ColE1 DNA in minicells by Meagher *et al.* (9). The plasmid pAO100 lacks about 25% of the gene required for expression of the plasmid mobilization (Fig. 3) (27, 31). Therefore, the appearance of 54,000-dalton peptide from pAO100 suggested that the direction of transcription of the relaxation protein is not clockwise, but counterclockwise. Polypeptides of 25,000, 17,000, and 15,000 daltons which were synthesized by pAO100 DNA, but not by pAO2 DNA may be fragments of the relaxation protein. Although the prominent band of 27,000-dalton polypeptide ap-

peared with pAO100 can not be assigned unequivocally, it is tentatively ascribed as a derivative of the 54,000-dalton polypeptide. A similar band was observed in the analysis of gene expression of the one-half plasmid in minicells (9).

A polypeptide electrophoresed near the front dye was absent in either *Eco*RI-cleaved ColE1 DNA, pAO100, or pAO2 (Fig. 2-b,d,e). The molecular weight was determined as 12,000, but it may be overestimated because of the position near front dye. This polypeptide was not a fragment of colicin E1, since the polypeptide could be found with *Sma*I-cleaved ColE1 in spite of the absence of majority of colicin E1 polypeptide (Fig. 2-c). The origin of this peptide must await further analysis.

Polypeptides of 44,000 and 41,000 daltons are probably encoded by the region of 0.23-0.44 map units of ColE1 plasmid, because these polypeptides did not react with colicin E1 antiserum and were not synthesized by pAO100 DNA. The 36,000, 33,000, 30,000, and 25,000-dalton polypeptides synthesized by *Sma*I-cleaved ColE1 DNA did not react with the colicin E1 antiserum, but the same molecular weight polypeptides synthesized by ColE1 DNA reacted with the antiserum (Fig. 2-a'). This fact indicates that the bands of these molecular weight polypeptides included fragments derived from colicin E1 protein and other unidentified polypeptides. Miller and Cohen (33) reported that an endonuclease having *Eco*RI specificity is produced by ColE1 plasmid. One of these unidentified polypeptides may be ascribed to the *Eco*RI endonuclease.

Taking into account of the size and the isotope incorporation into these polypeptides, the expression of genes for colicin E1 and immunity proteins was much more efficient than that for a relaxation protein. Since the direction of transcription of the immunity gene is clockwise (28) and those of the colicin E1 and the mobility genes are probably counterclockwise, the ColE1 plasmid appears to have at least three transcriptional units. This conclusion is not inconsistent with experiments on RNA polymerase binding sites on ColE1 DNA reported by Collins *et al.* (13).

ACKNOWLEDGEMENTS

We thank Dr. A. Oka for providing unpublished informations and Dr. K. Hayakawa for her suggestions on immunoprecipitation experiments. This work was supported by the Scientific Research Fund of the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Helinski, D.R. and Clewell, D.B. (1972) Ann. Rev. Biochem. 40, 899-942
2. Inselburg, J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2256-2259
3. Tomizawa, J., Sakakibara, Y. and Kakefuda, T. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 2260-2264
4. Hershfield, V., Boyer, H.W., Yanofsky, C., Lovett, M.A. and Helinski, D.R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3455-3459
5. Radman, M. (1974) in Molecular Mechanisms for Repair of DNA (Part A), Hanawalt, P.C. and Setlow, R.B. Eds., pp. 355-367, Plenum Press, New York.
6. Tessman, E.S., Gritzmacher, C.A. and Peterson, P.K. (1978) J. Bacteriol. 135, 29-38
7. Nakazawa, A. and Suzuki, N. (1977) J. Bacteriol. 131, 1011-1012
8. Dougan, G. and Sherrat, D. (1977) Mol. Gen. Genet. 15, 151-160
9. Meagher, R.B., Tait, R.C., Betlach, M. and Boyer, H.W. (1977) Cell 10, 521-536
10. Inselburg, J. and Applebaum, B. (1978) J. Bacteriol. 133, 1444-1451
11. Eichenlaub, R. (1974) J. Bacteriol. 120, 1476-1477
12. Yang, H. and Zubay, G. (1978) in Microbiology, Schlessinger, D. Ed., pp. 154-155, American Society for Microbiology, Washington, D.C.
13. Collins, J., Johnsen, M., Jørgensen, P., Valentin-Hansen, P., Karlström, H.O., Gautier, F., Lindenmaier, W., Mayer, H. and Sjöberg, B.M. (1978) in Microbiology, Schlessinger, D. Ed., pp. 150-153, American Society for Microbiology, Washington, D.C.
14. Nakazawa, A., Suzuki, N. and Tamada, T. (1977) Antimicrob. Agents. Chemother. 11, 219-224
15. Oka, A. (1978) J. Bacteriol. 133, 916-924
16. Clewell, D.B. and Helinski, D.R. (1970) Biochemistry 9, 4423-4440
17. Mandell, J.D. and Hershey, A.D. (1960) Anal. Biochem. 1, 66-77
18. Hinegardner, R.T. (1971) Anal. Biochem. 39, 197-201
19. Zubay, G., Chamoers, D.A. and Cheong, L.C. (1970) in The Lactose Operon, Beckwith, J.R. and Zipser, D. Eds., pp. 375-391, Cold Spring Harbor Laboratory, New York.
20. Korn, E.D. and Wright, P.L. (1973) J. Biol. Chem. 248, 439-447
21. Laemmli, U.K. (1970) Nature, 227, 680-685
22. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88
23. Schwartz, S.A. and Helinski, D.R. (1971) J. Biol. Chem. 246, 6318-6327
24. Ouchterlony, O. (1962) Progr. Allergy, 6, 30-154
25. Kessler, S.W. (1975) J. Immunol. 115, 1617-1624
26. Manley, J.L. (1978) J. Mol. Biol. 125, 407-432
27. Dougan, G., Saul, M., Warren, G. and Sherratt, D. (1978) Mol. Gen. Genet. 158, 324-327
28. Oka, A., Nomura, N., Morita, M., Sugisaki, H., Sugimoto, K. and Takanami, M. (1979) Mol. Gen. Genet. in press
29. Levine, A.D. and Rupp, W.D. (1978) in Microbiology, Schlessinger, D. Ed., pp. 163-166, American Society for Microbiology, Washington, D.C.
30. Watson, D.H. and Sherratt, D.J. (1979) Nature 278, 362-364
31. Hershfield, V., Boyer, H.W., Chow, L. and Helinski, D.R. (1976) J. Bacteriol. 126, 447-453
32. Lovett, M.A. and Helinski, D.R. (1975) J. Biol. Chem. 250, 8790-8795
33. Miller, C.A. and Cohen, S.N. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1265-1269