Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3601–3605, June 1985
Biochemistry

Regulation of methionine synthesis in *Escherichia coli*: Effect of *metJ* gene product and *S*-adenosylmethionine on the expression of the *metF* gene

(5,10-methylenetetrahydrofolate reductase/methionine repression)

ROBERT SHOEMAN*, BETTY REDFIELD*, TIMOTHY COLEMAN*, RONALD C. GREENE†, ALBERT A. SMITH†, NATHAN BROT*, AND HERBERT WEISSBACH*

*Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110; and †Veterans Administration Hospital, Durham, NC 27705

Contributed by Herbert Weissbach, February 5, 1985

ABSTRACT **The regulation of the expression of the *Escherichia coli metF* gene, which codes for 5,10-methylenetetrahydrofolate reductase (EC 1.1.99.15), has been investigated by using a simplified DNA-directed *in vitro* system that measures the formation of the first dipeptide (fMet-Ser) of the gene product. The synthesis of fMet-Ser directed by a plasmid containing the *metF* gene is specifically inhibited by *metJ* protein (repressor protein). *S*-Adenosylmethionine enhances the inhibition by the *metJ* protein of *metF* gene expression. The inhibition by the *metJ* protein is at the level of transcription and the results suggest that *S*-adenosylmethionine is functioning as an allosteric effector.**

The genes required for methionine synthesis in *Escherichia coli* are scattered throughout the chromosome (1), yet the synthesis of the proteins coded for by these genes is coordinately controlled by the level of methionine in the growth medium (2–6). Genetic and biochemical studies have indicated that the *metJ* gene product is a regulatory protein that is required for methionine repression of the methionine regulon (7–9). Since *metK* mutants, which are deficient in *S*-adenosylmethionine (AdoMet) synthetase (EC 2.5.1.6), also are regulatory mutants (10, 11), it appears that AdoMet, and not methionine, functions with the *metJ* gene product (repressor protein) to repress the methionine regulon. In addition, a second mechanism exists that specifically affects the expression of the *metF* (5,10-methylenetetrahydrofolate reductase, EC 1.1.99.15), and the *metE* (non-B_{12} N^5-methyltetrahydrofolate:homocysteine *S*-methyltransferase, EC 2.1.1.14) genes. Milner *et al.* (12) demonstrated that the synthesis of the *metE* and *metF* gene products in *E. coli* was repressed by the addition of vitamin B_{12} to the growth medium. The results were consistent with a mechanism in which the *metH* gene product (B_{12}-dependent methyltransferase, EC 2.1.1.13), which contains a cobalamin prosthetic group, was the functional repressor. The specificity of the vitamin B_{12} repression in these studies (only the *metE* and *metF* gene products were affected) indicated that the vitamin was not functioning by simply increasing the level of methionine in the cell due to activation of the B_{12}-dependent methyltransferase (12). Although the methionine and vitamin B_{12} repressions appear to involve different mechanisms, there are some data to suggest that the *metJ* protein is also involved in the vitamin B_{12} effect on the expression of the *metF* gene but not the *metE* gene (9). A summary of some of these reactions, including where methionine and vitamin B_{12} play a regulatory role, is shown in Fig. 1.

Recently, the *metF* gene has been cloned and sequenced (13). In the present report, we have used a plasmid containing the *metF* gene as template for *in vitro* studies on the regulation of the expression of this gene.

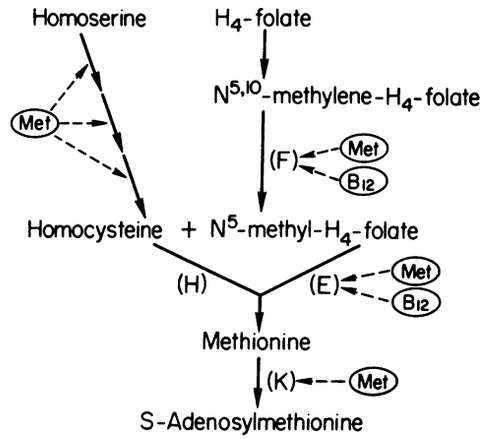

FIG. 1. Terminal reactions in methionine biosynthesis. Letters in parentheses refer to the genes that code for the different enzymes. F, 5,10-methylenetetrahydrofolate reductase; E, non-B_{12} methyltransferase; H, B_{12}-dependent methyltransferase; K, AdoMet synthetase. The genes whose expression is regulated by growth in the presence of methionine (Met) and vitamin B_{12} (B_{12}) are indicated.

MATERIALS AND METHODS

Materials. L-[^{3}H]Alanine, L-[^{3}H]valine, L-[^{3}H]serine, and L-[^{35}S]methionine were purchased from Amersham and [^{3}H]UTP was from ICN. Purified isoacceptor species $tRNA_3^{Ala}$, $tRNA^{Phe}$, $tRNA_1^{Ser}$, and $tRNA_3^{Ser}$ were purchased from Subriden RNA (Rolling Bay, WA) and purified $tRNA_f^{Met}$ was from Boehringer Mannheim. The tRNAs were aminoacylated with *E. coli* aminoacyl-tRNA synthetases [0.25 M salt eluate from a DEAE-cellulose column (14)] and fMet-$tRNA_f^{Met}$ was prepared as previously described (15, 16). The specific activities of the ^{3}H-aminoacylated tRNAs were 2500–4600 cpm/pmol. AdoMet was obtained from Sigma and was purified by ion-exchange chromatography on Dowex 1 HCO_3^- (17).

Plasmid pNF1337 (18), containing the ribosomal protein L10 operon, was originally supplied by J. Friesen (University of Toronto). Plasmid pRCG161, containing the *metF* gene, was constructed from the transducing phage λ d*met*141 (19). This phage carries a functional copy of the *metF* gene and a portion of the *metL* gene but lacks the *metJ* and *metB* genes. *Eco*RI/*Pst* I double digests of λ d*met*141 DNA and pBR322

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AdoMet, *S*-adenosylmethionine; EF-G, elongation factor G.

DNA were ligated with T4 DNA ligase. The ligation mixture was used to transform competent cells of strain RC709 (*metF63, pro-22*; obtained from Graham Walker, Massachusetts Institute of Technology, Cambridge, MA). pRCG161 was obtained from a tetracycline-resistant, ampicillin-sensitive, methionine-independent transformant.

The preparation and characterization of the purified *metJ* gene product will be described elsewhere. The protein used in the present experiments showed one major band on denaturing gels, corresponding to a molecular weight of 12,000 and behaved as a dimer in sedimentation equilibrium experiments under nondenaturing conditions. It was estimated to be greater than 90% pure (unpublished results). The concentration of the *metJ* protein was estimated by assuming that the A_{280} of a 1 mg/ml solution = 1.0. pRCG161 DNA was transcribed *in vitro* and the RNA was isolated as previously described (20), except that 20 μg of pRCG161 DNA and 30 μg of *E. coli* RNA polymerase were used in a 0.35-ml reaction mixture. The RNA was separated from any free nucleotides by five precipitations with ethanol containing 2 M ammonium acetate. A unit of RNA is defined as the amount of RNA that contains 1 pmol of incorporated [^{3}H]UTP under the conditions used (20).

DNA- and mRNA-Directed Dipeptide and Tripeptide Synthesis. The *in vitro* systems for DNA- and RNA-directed dipeptide and tripeptide synthesis have been previously described (21–25). Unless otherwise stated, each incubation mixture contained either 0.2 μg of pBR322 DNA, 2 μg of pRCG161 DNA, 1 μg of pNF1337 DNA, or 75 units of pRCG161 RNA (see above), and incubation was for 60 min at 37°C. The following dipeptides were synthesized: fMet-Ser, directed by both the *metF* and β-lactamase genes, utilizing Ser-tRNA$_3^{Ser}$; fMet-Ser, directed by the L12 gene, utilizing Ser-tRNA$_1^{Ser}$; and fMet-Ala, directed by the L10 gene (21). For dipeptide synthesis, the second amino acid was labeled with tritium and acylated to the appropriate isoacceptor tRNA species. For tripeptide synthesis, the appropriate unlabeled second aminoacyl-tRNA was used and the third ^{3}H-aminoacyl-tRNA and elongation factor G (EF-G) were added (22, 24). Unless otherwise indicated, *metJ* protein (0.4 μg/35-μl reaction mixture) and AdoMet (50 μM) were added at the beginning of the incubation.

The assay for the synthesis of the dipeptide and tripeptide products by using cation-exchange chromatography is described elsewhere (25).

DNA-Directed Protein Synthesis *in Vitro*. A highly defined *E. coli* DNA-directed *in vitro* protein synthesis system (26, 27) was also used for some of these studies. Each incubation mixture contained either 0.5 μg of pBR322 DNA or 1 μg of pRCG161 as template and the reaction was carried out for 60 min at 37°C. Radioactive material precipitable by hot Cl_3CCOOH was measured and aliquots of the incubation mixtures were applied to $NaDodSO_4$/15% polyacrylamide gels (28). After electrophoresis, the gels were soaked in EN^{3}HANCE (New England Nuclear), dried, and fluorographed at −70°C.

RESULTS

***In Vitro* Expression of the First Dipeptide of the *metF* Gene Product.** Plasmid pRCG161 was used as template in a simplified system that has been developed to measure the first di- or tripeptide of a gene product. The DNA sequence of the NH_2-terminal coding region of the *metF* gene is AUG-AGC-TTT (13), which corresponds to an initial tripeptide of (f)Met-Ser-Phe. Fig. 2 shows the effect of plasmid pRCG161 concentration on the formation of the NH_2-terminal dipeptide, fMet-Ser. Maximal synthesis is seen at levels of 3–4 μg of DNA and the reaction was linear for at least 60 min. The DNA sequence also predicts that tRNA$_3^{Ser}$ is the tRNA isoacceptor species required for dipeptide synthesis. As shown in Table 1, Ser-tRNA$_1^{Ser}$ could not replace Ser-tRNA$_3^{Ser}$ in dipeptide formation and an unrelated species, Ala-tRNA$_3^{Ala}$, was also inactive. Tripeptide synthesis was used to further substantiate that the *metF* gene was being expressed from pRCG161. As seen in Table 2, significant formation of fMet-Ser-Phe is obtained that is dependent on both EF-G and Ser-tRNA$_3^{Ser}$. Because of the simplicity of the dipeptide system, it was used initially to study the regulation of the expression of the *metF* gene product.

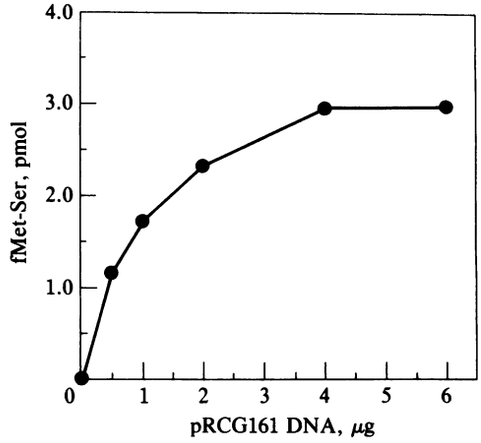

FIG. 2. Effect of plasmid pRCG161 DNA concentration on dipeptide synthesis. Details are described in the text.

Effect of the *metF* Gene Product and AdoMet on Expression of the *metF* Gene. Genetic and biochemical studies have indicated that the *metJ* gene product and AdoMet are involved in the repression of the methionine biosynthetic pathway (7–11). Therefore, the purified *metJ* gene product and AdoMet were tested in the dipeptide system for their effect on the expression of the *metF* gene from plasmid pRCG161, the β-lactamase gene from plasmid pBR322, and the ribosomal protein L10 and L12 genes from plasmid pNF1337. AdoMet alone, at concentrations up to 100 μM, had no effect on dipeptide synthesis (data not shown). The *metJ* protein, in the presence of AdoMet, inhibited *metF* gene expression (Fig. 3*A*) but had no significant effect on β-lactamase expression or on the expression of the L10 and L12 genes (data not shown). In all of these experiments, AdoMet was present at a concentration of 50 μM but, as shown in Fig. 3*A*, the requirement for AdoMet is dependent on the amount of *metJ* protein present. At low concentrations (<0.5 μg per incubation) of the *metJ* protein, the addition of AdoMet markedly increases the inhibitory effect of the *metJ* protein. However, higher levels of the *metJ* protein alone (>1.5 μg per incubation) can significantly inhibit fMet-Ser formation directed by pRCG161. The ability of AdoMet to enhance the effect of low levels of the *metJ* protein on *metF* gene expression was dependent on the concentration of AdoMet (Fig. 3*B*). Concentrations of AdoMet above 10 μM were

Table 1. Dipeptide synthesis directed by plasmid pRCG161 DNA

Aminoacyl-tRNA isoacceptor species	Dipeptide, pmol
[^{3}H]Ser-tRNA$_3^{Ser}$	2.8
[^{3}H]Ser-tRNA$_1^{Ser}$	0.06
[^{3}H]Ala-tRNA$_3^{Ala}$	0.01

The incubations for dipeptide synthesis were performed as described elsewhere (22–24). Where indicated, 8–10 pmol of the individual aminoacylated tRNA species was used.

Table 2. Di- and tripeptide synthesis directed by the *metF* gene from plasmid pRCG161 DNA

Omission	Peptide, pmol	
	fMet-Ser	fMet-Ser-Phe
None	2.7	0.7
Ser-tRNA$_3^{Ser}$	—	0.02
EF-G	—	0.03

The complete system for dipeptide formation contained 2 μg of pRCG161 DNA and [^{3}H]Ser-tRNA$_3^{Ser}$ (2500 cpm/pmol). The complete system for tripeptide formation contained Ser-tRNA$_3^{Ser}$ (unlabeled), [^{3}H]Phe-tRNA^{Phe} (4600 cpm/pmol), and 0.5 μg of EF-G in addition to the other components for dipeptide synthesis (23).

required to obtain a significant effect, and *S*-adenosylhomocysteine (50 μM) could not replace AdoMet in this system, nor did it inhibit the AdoMet-mediated action of the *metJ* protein (data not shown). It should be noted that different preparations of the *metJ* protein all gave similar results in that high concentrations of the protein inhibited *metF* expression in the absence of AdoMet, whereas lower amounts of *metJ* protein required AdoMet for maximal inhibition. However, each batch of *metJ* protein had to be titrated separately to obtain the level that gave the optimal stimulation by AdoMet.

Initial experiments performed at various Mg^{2+} concentrations yielded variable results, suggesting that the effect of the *metJ* protein and AdoMet on fMet-Ser formation directed by pRCG161 was also dependent on the concentration of Mg^{2+}. As seen in Fig. 4, at Mg^{2+} concentrations above 13 mM the *metJ* protein has little effect on dipeptide synthesis. Routinely, the incubation mixtures for dipeptide synthesis contained between 9 and 10.5 mM Mg^{2+}, which appears optimal for both dipeptide synthesis and the *metJ* protein inhibitory effect.

Effect of the *metJ* Gene Product on Transcription. As seen in Table 3, the *metJ* protein inhibited the expression of the *metF* gene from the plasmid template but not from the mRNA prepared from that plasmid (see *Materials and Methods*). These results indicate that the *metJ* protein is affecting transcription of the *metF* gene, or specifically inactivating the DNA template. To test for a specific nuclease activity associated with the *metJ* protein, aliquots of plasmid pRCG161 DNA were incubated in the dipeptide system in the presence and absence of *metJ* protein and were subjected to agarose gel electrophoresis. No evidence of nuclease activity was found (results not shown).

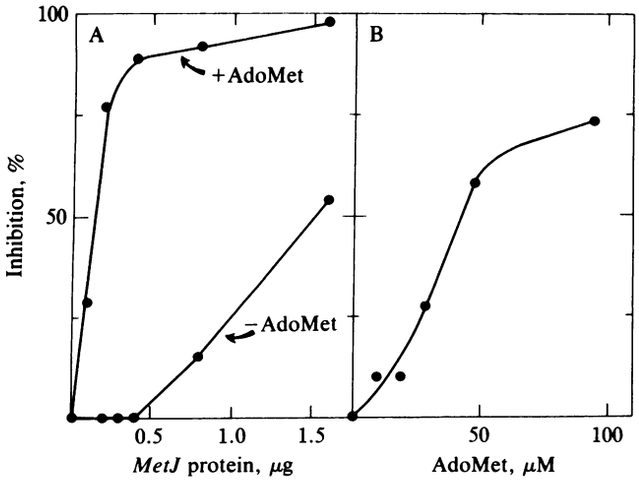

FIG. 3. Effect of AdoMet and *metJ* protein on the inhibition of fMet-Ser formation from plasmid pRCG161. (*A*) Where indicated, AdoMet was added at a final concentration of 50 μM. (B) *metJ* protein (0.4 μg) was added to the incubation mixture. In the absence of *metJ* protein and AdoMet, 2.7 pmol of fMet-Ser was synthesized.

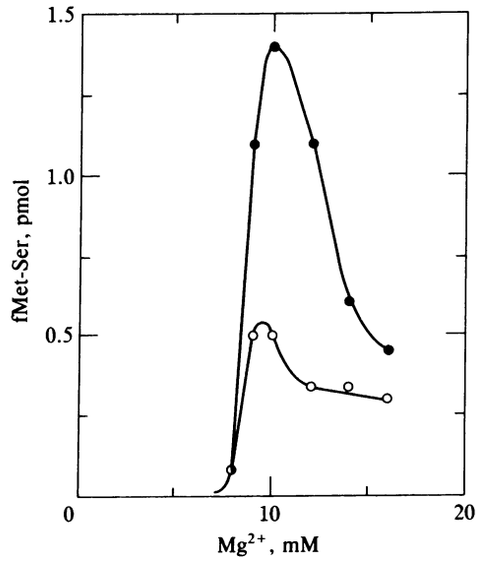

FIG. 4. Effect of Mg^{2+} on dipeptide synthesis and *metJ* repression of *metF* gene expression. Dipeptide synthesis was performed in the absence (●) or presence (○) of *metJ* protein and AdoMet, as described in the text except that the Mg^{2+} concentration was varied as indicated. Each incubation mixture contained 2 μg of pRCG161 DNA and, where indicated, 0.4 μg of *metJ* protein and 50 μM AdoMet.

Synthesis of the *metF* gene product *in Vitro*. A defined *in vitro* system was used to demonstrate synthesis of the entire *metF* gene product and to confirm the results that were obtained with the *metJ* protein and AdoMet in the dipeptide system. Fig. 5 shows a gel of the products labeled with [^{35}S]methionine *in vitro*, using either pBR322 or pRCG161 as template. With the former template, the major product *in vitro* is β-lactamase, molecular weight ≈32,000 (lane 3), whereas with the *metF* plasmid, a protein that migrates with an apparent molecular weight of ≈31,000 is synthesized (lane 5). The latter is in reasonable agreement with the calculated molecular weight of 33,000 for the *metF* gene product (13). As also seen in Fig. 5, addition of *metJ* protein and AdoMet markedly inhibits the synthesis of the *metF* protein (lane 6) but has no effect on the synthesis of β-lactamase (lane 4).

DISCUSSION

There appear to be two distinct mechanisms that are involved in the regulation of the methionine biosynthetic pathway in *E. coli*. With the exception of the B_{12}-dependent methyltransferase (*metH* gene product), all of the genes in this regulon are repressed by the presence of methionine in the growth

Table 3. Inhibition of *metF* gene expression by the *metJ* protein with DNA and RNA templates

Template	Additions to dipeptide system	Dipeptide (fMet-Ser), pmol
pRCG161 DNA	None	2.7
	metJ protein, AdoMet	0.8
pRCG161 RNA	None	0.6
	metJ protein, AdoMet	0.6

The dipeptide system contained 2 μg of plasmid DNA or 75 units of RNA as template. *metJ* protein (1.5 μg) and AdoMet (10 μM final concentration) were added as indicated. RNA polymerase was omitted from the incubation mixtures containing mRNA.

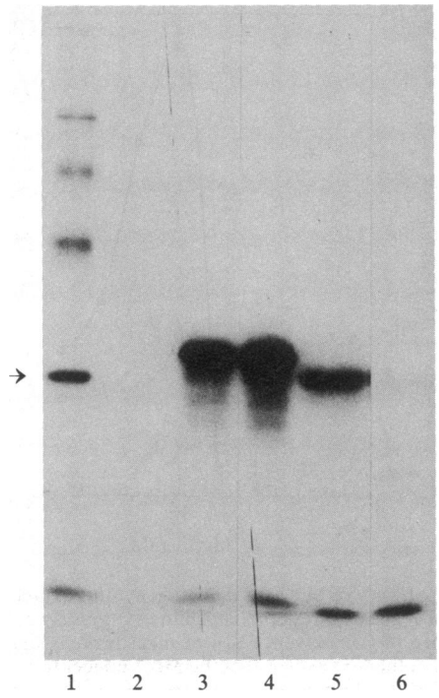

FIG. 5. NaDodSO$_4$/polyacrylamide gel electrophoresis of [^{35}S]-methionine-labeled proteins produced in a defined system *in vitro*. Lane 1, protein standards; lane 2, minus DNA; lane 3, pBR332 DNA; lane 4, pBR322 plus *metJ* protein and AdoMet; lane 5, pRCG161; lane 6, pRCG161 plus *metJ* protein and AdoMet. Where indicated, 3 μg of *metJ* protein and 10 μM AdoMet were added. The position of the *metF* gene product is marked with an arrow. Aliquots containing about 50,000 cpm of Cl_3CCOOH-insoluble protein were subjected to electrophoresis in lanes 3–6.

medium (2-6, 9, 12). There are two known classes of regulatory mutants of methionine biosynthesis, referred to as *metJ* and *metK* mutants (7–11). Since the *metK* gene product is responsible for the synthesis of AdoMet (7, 9), one could picture the *metJ* gene product as either a repressor protein that requires AdoMet for activity or a methyltransferase that accepts or transfers a methyl group from AdoMet.

The availability of a cloned *metF* gene and purified *metJ* protein has enabled us to investigate the *in vitro* expression and regulation of the *metF* gene. For these studies, we primarily have used a simplified *in vitro* system that measures the formation of the first dipeptide of the gene product, although these results have also been confirmed in a defined *in vitro* system that is capable of synthesizing the entire gene product. A specific inhibition by the *metJ* protein and AdoMet on the expression of the *metF* gene has been observed. Although high levels of the *metJ* protein alone can inhibit *metF* gene expression, AdoMet markedly stimulated the inhibition when low levels of the *metJ* protein were used. The inhibition observed was at the level of transcription, supporting the view that the *metJ* protein is a repressor. However, the role of AdoMet in this process is still not clear. As noted above, the ability of AdoMet to enhance the *metJ* protein effect was greatest at low *metJ* protein concentrations, but it did vary with different preparations of the *metJ* protein. For example, one batch of *metJ* protein showed significant inhibition of *metF* gene expression even when added at low levels (<0.5 μg per incubation), and AdoMet caused only a 2-fold enhancement of the effect. Other preparations of *metJ* protein showed essentially no effect when present at low levels unless AdoMet was present (see Fig. 3*A*). One explanation for these results might be that the isolated *metJ* protein is a mixture that consists of a methylated (active) and nonmethylated (inactive) species. If so, the lack of a complete dependency on AdoMet as well as the variation in the activity of the *metJ* protein could be explained if each preparation of the *metJ* protein varied in the extent of methylation. Preliminary attempts to show a transfer of a methyl group from AdoMet to the *metJ* protein or the plasmid DNA have been unsuccessful. The fact that *S*-adenosylhomocysteine, known to be an inhibitor of AdoMet-dependent methyltransferases (29), had no effect in this system provides additional indirect evidence that the *metJ* protein is not functioning simply as a methyltransferase. Another possibility is that a AdoMet·*metJ* protein complex is the active repressor and that the different *metJ* preparations may contain various amounts of tightly bound AdoMet. However, a stable binding of AdoMet to the *metJ* protein could not be demonstrated and the A_{280}/A_{260} ratio of the purified protein was 1.7, which argues against the presence of AdoMet bound to the protein. At present, the results favor the view that AdoMet is acting as an allosteric effector for the *metJ* protein. The plot of inhibition as a function of AdoMet concentration showed a sigmoidal curve (Fig. 3*B*), which would be consistent with an allosteric mechanism. It is clear that further information is needed on the structure of the purified *metJ* protein and its ability to interact with the *metF* regulatory region to elucidate how AdoMet is functioning in this system.

1. Bachmann, B. J. & Low, K. B. (1980) *Microbiol. Rev.* **44,** 1–56.
2. Cohn, M., Cohen, G. N. & Monod, J. (1953) *C.R. Hebd. Seances Acad. Sci. Paris* **236,** 746–748.
3. Wijesundera, S. & Woods, D. D. (1953) *Biochem. J.* **55,** viii.
4. Cohen, G. N. & Jacob, F. (1953) *C.R. Hedb. Seances Acad. Sci. Paris* **248,** 3490–3492.
5. Rowberg, R. J. (1965) *Nature (London)* **206,** 962.
6. Patte, J. C., Le Bras, G. & Cohen, G. N. (1967) *Biochim. Biophys. Acta* **136,** 245–257.
7. Holloway, C. T., Greene, R. C. & Su, C.-H. (1970) *J. Bacteriol.* **104,** 734–747.
8. Su, C.-H. & Greene, R. C. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 367–371.
9. Kung, H.-F., Spears, C., Greene, R. C. & Weissbach, H. (1972) *Arch. Biochem. Biophys.* **150,** 23–31.
10. Greene, R. C., Su, C.-H. & Holloway, C. T. (1970) *Biochem. Biophys. Res. Commun.* **38,** 1120–1126.
11. Greene, R. C., Hunter, J. S. V. & Coch, E. H. (1973) *J. Bacteriol.* **115,** 57–67.
12. Milner, L., Whitfield, C. & Weissbach, H. (1969) *Arch. Biochem. Biophys.* **133,** 413–419.
13. Saint-Girons, I., Duchange, N., Zakin, M. M., Park, I., Margarita, D., Ferrara, P. & Cohen, G. N. (1983) *Nucleic Acids Res.* **11,** 6723–6732.
14. Kung, H. F., Spears, C. & Weissbach, H. (1975) *J. Biol. Chem.* **250,** 1556–1562.
15. Dickerman, H. W., Steers, E., Jr., Redfield, B. G. & Weissbach, H. (1967) *J. Biol. Chem.* **242,** 1522–1528.
16. Schofield, P. (1970) *Biochemistry* **9,** 1694–1700.
17. Shapiro, S. K. & Ehninger, D. J. (1966) *Anal. Biochem.* **15,** 323–333.
18. Fiil, N. P., Bendiak, D., Collins, J. & Friesen, J. D. (1979) *Mol. Gen. Genet.* **173,** 39–50.
19. Krueger, J. H., Johnson, J. R., Greene, R. C. & Dresser, M. (1981) *J. Bacteriol.* **147,** 612–621.
20. Peacock, S., Brot, N. & Weissbach, H. (1983) *Biochem. Biophys. Res. Commun.* **113,** 1018–1025.
21. Robakis, N., Meza-Basso, L., Brot, N. & Weissbach, H. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4261–4264.
22. Cenatiempo, Y., Robakis, N., Reid, B. R., Weissbach, H. & Brot, N. (1982) *Arch. Biochem. Biophys.* **218,** 572–578.
23. Cenatiempo, Y., Robakis, N., Meza-Basso, L., Brot, N., Weissbach, H. & Reid, B. R. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1466–1468.
24. Robakis, N., Cenatiempo, Y., Meza-Basso, L., Brot, N. & Weissbach, H. (1983) *Methods Enzymol.* **101,** 690–706.

25. Peacock, S., Weissbach, H. & Nash, H. A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6009–6013.
26. Kung, H.-F., Redfield, B., Treadwell, B. V., Eskin, B., Spears, C. & Weissbach, H. (1977) *J. Biol. Chem.* **252,** 6889–6894.
27. Zarucki-Schulz, T., Jerez, C., Goldberg, G., Kung, H.-F., Huang, K.-H., Brot, N. & Weissbach, H. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 6115–6119.
28. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
29. Cantoni, G. L., Richard, H. H. & Chiang, P. K. (1979) *Transmethylation*, eds. Usdin, E., Borchardt, R. T. & Creveling, C. R. (Elsevier/North-Holland, New York), pp. 155–164.