Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 32, Issue of August 6, pp. 22143–22146, 1999

Formylation Is Not Essential for Initiation of Protein Synthesis in All Eubacteria*

(Received for publication, May 20, 1999)

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Formylation of the initiator methionyl-tRNA, catalyzed by methionyl-tRNA formyltransferase, has long been regarded as essential for initiation of protein synthesis in eubacteria. Here, we show that this process is, in fact, dispensable in *Pseudomonas aeruginosa*. Disruption of the chromosomal methionyl-tRNA formyltransferase gene in *P. aeruginosa* resulted only in a moderate decrease in the rate of cell growth, whereas in *Escherichia coli* cell growth was severely impaired. The ability of the *P. aeruginosa* mutant strain to grow was not due to an additional copy of the methionyl-tRNA formyltransferase gene or to *N*-acylation of the methionyl moiety by a group other than formyl. These results indicate that *P. aeruginosa* can carry out formylation-independent initiation of protein synthesis, using the nonformylated methionyl-tRNA. Therefore, the dogma that eubacteria require formylation of the initiator methionyl-tRNA for initiation of protein synthesis may have been an invalid generalization of results obtained with *E. coli*.

Protein synthesis in eubacteria, and in the chloroplasts and mitochondria of eukaryotes, can be initiated using the initiator formyl-methionyl-tRNA (fMet-tRNA^{fMet})[1] (1). Formylation is specific for the initiator methionyl-tRNA (Met-tRNA^{fMet}) and is catalyzed by methionyl-tRNA formyltransferase (MTF), which is encoded by the *fmt* gene (2–5). The features in the *Escherichia coli* tRNA^{fMet} required for formylation are the base-base mismatch between nucleotides 1 and 72 and the second and third base pairs of the acceptor stem (4–6). The fMet moiety allows initiation factor IF-2 to recognize the initiator tRNA and reject other tRNAs. This conclusion is based on *in vivo* analysis of the effect of overproduction of IF-2 on the activity of *E. coli* tRNA^{fMet} mutants defective in formylation (7, 8) and on *in vitro* studies of IF-2 interaction with *N*-blocked aminoacyl-tRNA (9, 10). The formyl group also prevents the tRNA^{fMet} from participating in elongation by blocking binding of elongation factor EF-Tu (5, 7).

Formylation of the Met-tRNA^{fMet} is generally accepted as a key checkpoint required for initiation of protein synthesis in eubacteria. This dogma, based primarily on studies conducted in *E. coli*, was further substantiated by the finding that disruption of the *E. coli* chromosomal *fmt* gene severely curtailed cell growth (3). However, some earlier studies have obtained circumstantial evidence that questioned this generalization. A *Streptococcus faecalis* folate-deficient strain, which is unable to synthesize the formyl donor N^{10}-formyltetrahydrofolate (fTHF), was shown to be unaffected in growth or viability (11). However, it was not clear that *S. faecalis* can initiate protein synthesis in the absence of formylation, since the strain also incurred additional chromosomal mutations that affect proper tRNA modification (11).

While the studies in *S. faecalis* were inconclusive, they hinted that formylation may not be a prerequisite for initiation of protein synthesis in all eubacteria. Therefore, we investigated the significance of the formylation step in *Pseudomonas aeruginosa*, a Gram-negative, opportunistic pathogen that is distantly related to *E. coli*. The results show that the process of initiation of protein synthesis in *P. aeruginosa* is not strictly dependent on formylation and can accommodate the use of non-*N*-blocked Met-tRNA^{fMet}. The extent to which other eubacteria have this capacity to initiate translation thus becomes an open question.

EXPERIMENTAL PROCEDURES

Materials—The plasmids pEX100T (12), pUCGM (13), and pUCP26 (14) and the *P. aeruginosa* PAO1 strain were provided by Dr. J. S. Lam, Department of Microbiology, University of Guelph. The pACTN vector was described previously (15). The *E. coli* methionyl-tRNA synthetase (MetRS) and initiator tRNA (15) and *P. aeruginosa* MTF (15, 16) were overproduced and purified as described. The *fmt*-deficient strain of *E. coli* was constructed previously (16).

Construct for Expression of the P. aeruginosa and E. coli MTFs in the E. coli fmt Mutant Strain—The open reading frame (ORF) of the *fmt* genes was cloned into the *Nco*I and *Bam*HI sites in pACTN (15). In this construct, the *fmt* genes are under the control of the *Lac* promoter and the Shine-Dalgarno and transcription termination sequences of *gene 10* of bacteriophage T7.

Construct for Expression of the P. aeruginosa MTF in the P. aeruginosa fmt Mutant Strain—A 1.4-kilobase pair *Kpn*I fragment containing the wild type *P. aeruginosa fmt* gene was inserted into the same site in the *P. aeruginosa* shuttle vector pUCP26. The cloned fragment is under the control of the *Lac* promoter.

Disruption of the Chromosomal P. aeruginosa fmt Gene—The *P. aeruginosa fmt* gene on a 6-kilobase pair fragment was isolated previously from a genomic library (GenBank™ accession number AF073952) (16). A 4.5-kilobase pair *Sma*I segment containing the *fmt* gene and flanking sequences was obtained from the 6-kilobase pair fragment and inserted into the same site of pEX100T. An 840-base pair fragment containing the gentamycin resistance gene (Gm^R) was obtained by PCR amplification using pUCGM as the template. The fragment was digested with *MfeI* and inserted into the same site in the *fmt* gene on pEX100T. The pEX100T*fmt*::Gm^R construct was used to replace the chromosomal MTF gene of the *P. aeruginosa* PAO1 strain (12). Gene replacement was verified by PCR analysis using primers complementary to the 5′ and 3′ ends of the *fmt* ORF. The sequence of the primers are 5′-TTTTCCATGGCGATGAGCCAAGCATTGCGC-3′ and 5′-TTTGGATCCTCATTGGCCGAGCACCTG-3′.

Preparation of Cell Extracts—The wild type and mutant *fmt P. aeruginosa* strains harboring the pUCP26 vector without the *fmt* gene were grown overnight at 37 °C in Luria-Bertani medium supplemented with 60 μg/ml tetracycline. An aliquot of the cultures were diluted

* This work was supported by a research grant from the Natural Sciences and Engineering Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: fMet-tRNA^{fMet}, formyl-methionyl-tRNA; Met-tRNA^{fMet}, methionyl-tRNA; tRNA^{fMet}, initiator tRNA; MTF, methionyl-tRNA formyltransferase; ORF, open reading frame; fTHF, N^{10}-formyltetrahydrofolate; IF-2, initiation factor IF-2.

50-fold into 3 ml of medium containing tetracycline and grown for 3 h at 37 °C. Cells from 1.2 ml of culture was pelleted by centrifugation and lysed (8). The cell lysate was centrifuged and an aliquot of the supernatant was mixed with 3 volumes of 25.6 mM Tris-HCl, pH 8.0, 13.3 mM β-mercaptoethanol, 200 mM KCl, and 66% glycerol (w/v) and stored at −20 °C (8).

Measurement of MTF Activity—The initiator tRNA substrate was acylated with methionine at 37 °C for 30 min. The incubation mixture (20 μl) consisted of 20 mM imidazole HCl, pH 7.5, 150 mM NH_4Cl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/ml bovine serum albumin, 100 μM methionine (specific activity: 13,000–15,000 cpm/pmol), 2 mM ATP, 5 μM $tRNA^{fMet}$, and an excess of MetRS (1 μg). To measure the formylation activity in cell extracts, 5 μl of 1.8 mM of fTHF in 20 mM imidazole HCl, pH 7.5, and 5 μl of appropriately diluted MTF were added, and the incubation mixture was incubated for 10 min at 37 °C. The reaction was terminated by the addition of 30 μl of 360 mM copper sulfate in 1.1 M Tris, pH 7.3. After 5 min an aliquot was spotted on Whatman No. 3MM filter paper and washed sequentially with 10% trichloroacetic acid containing 0.2% casamino acids, 5% trichloroacetic acid containing 0.2% casamino acids, and cold 95% ethanol. The retained radioactivity was determined by liquid scintillation counting.

Determination of the Amount of N-Blocked Met-tRNA^{fMet} in the Wild Type and Mutant fmt P. aeruginosa Strains—The wild type and mutant strains carrying the pUCP26 vector were grown at 37 °C in 100 ml of Luria-Bertani medium containing 60 μg/ml tetracycline to an A_{600} of 1. The cells were pelleted and resuspended in 1 ml of 300 mM sodium acetate, pH 4.8, buffer containing 10 mM NaEDTA (3). Total tRNA was extracted from the cells with phenol and purified under acidic conditions at 4 °C (3). A portion of the tRNA samples was completely deacylated by a 90-min incubation at 37 °C in 0.5 ml of 2 M Tris-HCl, pH 8.0. Cleavage of the ester linkage of aminoacyl-tRNAs in another portion of the samples was carried out for 30 min at 37 °C in 0.5 ml of 200 mM sodium acetate, pH 5.0, buffer containing 10 mM $CuSO_4$. 0.5 A_{260} units of tRNA from the fully deacylated and $CuSO_4$-treated samples were acylated with methionine for 5 min using a large excess of MetRS. Formylation was carried out for 5 min using a large excess of affinity purified *P. aeruginosa* MTF. The amount of *N*-blocked Met-$tRNA^{fMet}$ in each strain was determined by the difference between formyl acceptance of the two tRNA samples and expressed as a percent of the total $tRNA^{fMet}$ in the completely deacylated sample.

RESULTS AND DISCUSSION

In vitro studies of protein synthesis using extracts from E. coli showed that translation was stimulated by fMet-$tRNA^{fMet}$ (17). Furthermore, inhibition of formylation in *E. coli* by using trimethoprim to impair folate metabolism caused the cells to grow very slowly (18–20). These early studies led to the belief that formylation is an important prerequisite for initiation of protein synthesis in eubacteria. Recently, this view was supported by the finding that inactivation of the *E. coli* chromosomal *fmt* gene, which codes for the formylating enzyme, severely curtailed cell growth (3). Both *in vitro* and *in vivo* studies showed that the formyl group is important for selection of the initiator tRNA by IF-2 at the initiation site of the ribosome and for preventing misappropriation of the tRNA in elongation (5, 7–10).

Some circumstantial evidence obtained from studies conducted in *S. faecalis* and *Bacillus subtilis* questioned the importance of formylation in initiation of protein synthesis in eubacteria. In contrast to *E. coli*, *B. subtilis* was capable of growth when formylation was blocked by trimethoprim inhibition of folate metabolism (21). Similarly, a *S. faecalis* mutant deficient in folate synthesis was not affected in growth (11). In the case of *S. faecalis*, the selection conditions used to isolate the mutant strain resulted in additional chromosomal mutations, which affected proper tRNA modification. This observation suggests that growth of *B. subtilis* could be due to compensatory chromosomal mutations. Therefore, it is not clear whether formylation is required for initiation of protein synthesis in these organisms.

Disruption of the P. aeruginosa Chromosomal MTF Gene—To investigate the importance of Met-$tRNA^{fMet}$ formylation in *P. aeruginosa*, the chromosomal *fmt* gene was replaced with a mutant copy by homologous recombination (12). The *fmt* gene used in this study was isolated previously from a genomic library by functional complementation of a MTF-deficient *E. coli* strain (16). A 4.5-kilobase pair *Sma*I segment containing the *fmt* gene as well as flanking sequences was obtained from the genomic clone and inserted into the same site in the suicide vector pEX100T. The *fmt* gene was inactivated by insertion of the Gm^R cassette into a *MfeI* site. *P. aeruginosa* transformants containing the pEX100T*fmt*::Gm^R vector in the chromosome were identified by selecting for carbenicillin and gentamycin resistance. Resolution of the plasmid was achieved by subjecting the merodiploid strain to sucrose counter-selection in the presence of gentamycin.

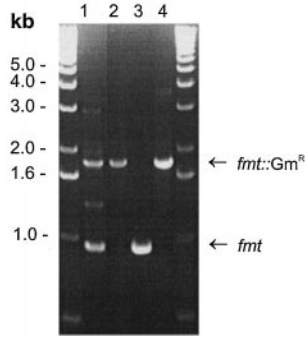

FIG. 1. **Verification that the *P. aeruginosa* chromosomal *fmt* gene was disrupted.** Replacement of the chromosomal *fmt* gene with the mutant copy was verified by PCR analysis using primers complementary to the 5′ and 3′ ends of the *fmt* ORF and chromosomal DNA from the merodiploid (*lane 1*), MTF mutant (*lane 2*), and wild type (*lane 3*) strains. The pEX100*fmt*::Gm^R DNA was used as a control (*lane 4*).

Several sucrose- and gentamycin-resistant colonies were observed within 24 h of incubation at 37 °C. Replacement of the wild type *fmt* gene with the mutant copy was verified by PCR analysis using primers complementary to the 5′ and 3′ ends of the *fmt* ORF (Fig. 1). Two products of about 1.7- and 1-kilobase pair, corresponding to the *fmt*::Gm^R fragment (compare *lanes 1* and *4*) and the wild type *fmt* ORF (compare *lanes 1* and *3*), respectively, were obtained when the chromosomal DNA from the merodiploid strain was used (*lane 1*). The presence of the *fmt*::Gm^R fragment and absence of the wild type *fmt* gene confirmed replacement of the chromosomal copy in one of the isolates (*lane 2*).

Formylation Is Less Important in P. aeruginosa than in E. coli—The *P. aeruginosa* MTF mutant strain grew with a doubling time of 152 min (Fig. 2*A*). Complementation with the wild type *fmt* gene, provided in trans on the plasmid pUCP26, decreased the doubling time of the mutant strain to 56 min. This is comparable with that of the wild type strain harboring the pUCP26 vector with or without the *fmt* gene (Fig. 2*A*). This finding established that the reduced growth rate of *P. aeruginosa* was due specifically to inactivation of the chromosomal *fmt* gene. The *E. coli* MTF mutant, constructed as described previously (3, 16, 22), doubled every 474 min, whereas the mutant strain expressing the wild type *E. coli* or *P. aeruginosa* *fmt* ORFs grew with a doubling time of 42–46 min (Fig. 2*B*). The residual growth of the *E. coli* mutant strain is probably due to some low level utilization of the unformylated Met-$tRNA^{fMet}$ in initiation. The growth rate of the MTF-deficient *E. coli* strain was reduced by 12-fold and that of the *P. aeruginosa* mutant strain was reduced by 3-fold. The distinct growth phenotype of the two mutant strains suggests that formylation is less important in *P. aeruginosa* than in *E. coli*.

Growth of the *P. aeruginosa fmt* mutant strain was not due to the presence of a homologue of MTF or to the mutant MTF enzyme being active. This conclusion is based on measurement

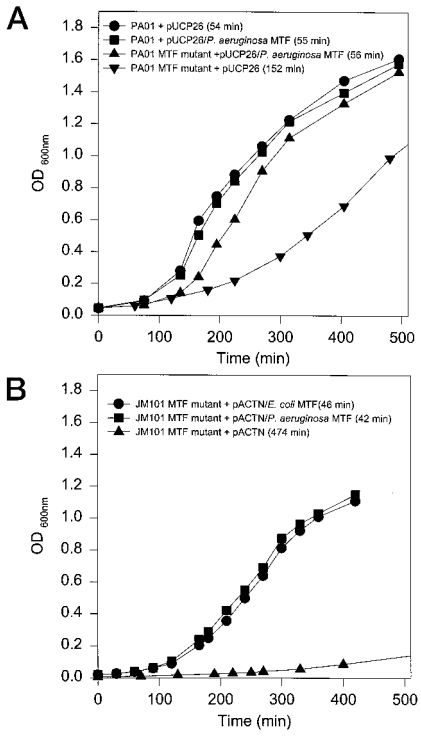

FIG. 2. **Growth phenotype of the *P. aeruginosa* and *E. coli* MTF mutant strains.** *A*, the *P. aeruginosa* PAO1 strains harboring the pUCP26 vector without (●, ▼) and with the *P. aeruginosa fmt* gene (■, ▲) were grown at 37 °C in Luria-Bertani medium containing 60 μg/ml tetracycline. *B*, the *E. coli* MTF-deficient strain harboring the pACTN vector without (▲) and with the *E. coli* (●) or *P. aeruginosa* (■) *fmt* gene was grown at 37 °C in 100 ml of Luria-Bertani medium supplemented with 100 μg/ml ampicillin, 10 μg/ml tetracycline, 25 μg/ml kanamycin, and 1 mM isopropyl-β-D-thiogalactopyranoside. At the times indicated the optical density of the cultures was measured at 600 nm. The doubling time for each strain is shown in the *inset*.

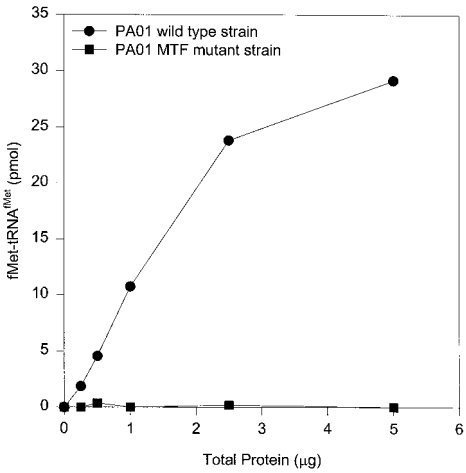

FIG. 3. **Formylating activity in cell extract prepared from the wild type and mutant *fmt P. aeruginosa* strains.** Cell extracts were prepared from the wild type (●) and mutant (■) strains carrying the pUCP26 vector alone. The formylation activity in each extract was measured as described under "Experimental Procedures."

of formylation activity in cell extracts prepared from the wild type and mutant strains (Fig. 3). The total activity in cell extracts prepared from the wild type parental strain increased with the amount of protein assayed. In contrast, no formylation activity was detected in cell extracts prepared from the mutant strain even at high protein levels. These results show that the

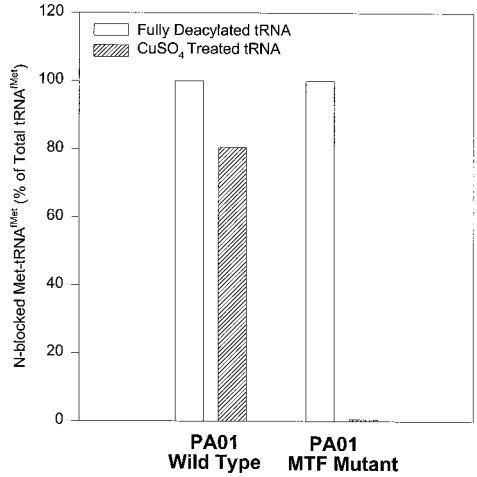

FIG. 4. **Determination of the amount of *N*-blocked Met-tRNA^{fMet} in the *P. aeruginosa* MTF-deficient strain.** The amount of *N*-blocked Met-tRNA^{fMet} in the wild type and mutant MTF strains was determined by the difference between formyl acceptance of the completely deacylated and $CuSO_4$-treated tRNA samples and expressed as a percent of the total tRNA^{fMet} in the completely deacylated sample (see "Experimental Procedures"). The data reported are averages of assays on three independent preparations.

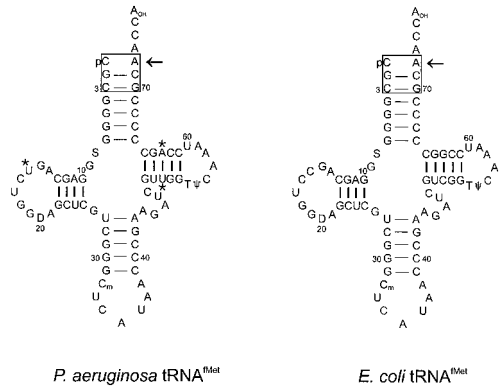

FIG. 5. **Nucleotide sequences of the *P. aeruginosa* and *E. coli* initiator tRNAs.** The *P. aeruginosa* initiator tRNA sequence was obtained by a BLAST search of the National Center for Biotechnology Information *P. aeruginosa* data base for sequences homologous to that of the *E. coli* initiator tRNA. The *boxes* indicate the determinants that are important for MTF recognition of the initiator tRNA. The *arrows* denote the most crucial of the three formylation determinants. The *asterisks* signify the nucleotides that are different between the *P. aeruginosa* and *E. coli* tRNAs.

P. aeruginosa fmt mutant strain was devoid of formylating activity.

P. aeruginosa Has the Capacity to Initiate Protein Synthesis with Non-N-blocked Met-tRNA^{fMet}—We investigated whether *N*-acylation of the Met-tRNA^{fMet} by a functional group other than the formyl group could account for the growth of the *P. aeruginosa fmt* mutant strain. Total tRNAs were isolated from the wild type and mutant strains, under conditions that stabilize the aminoacyl ester linkage (3). One portion of the sample was completely deacylated, by incubating the tRNA under alkaline conditions. A second portion was treated with $CuSO_4$, which cleaves the ester bond of aminoacyl-tRNA but not that of *N*-acyl-Met-tRNA^{fMet}. Both tRNA samples were re-acylated with methionine, and formyl acceptance was measured using *P. aeruginosa* MTF overproduced in *E. coli* and purified by affinity chromatography (15). The amount of *N*-blocked Met-tRNA^{fMet} in the wild type and mutant MTF strains was determined by the difference between formyl acceptance of the completely deacylated and $CuS0_4$-treated tRNA samples. Based on

this analysis, 80% of the Met-tRNA^{fMet} in the wild type strain was *N*-blocked (Fig. 4). However, only the unblocked Met-tRNA^{fMet} species was found in the mutant strain. This result showed that the Met-tRNA^{fMet} in the MTF-deficient strain was not *N*-acylated by another group. Taken altogether, the results indicate that protein synthesis in the MTF-deficient *P. aeruginosa* strain is initiated with the initiator methionyl-tRNA and not with *N*-blocked Met-tRNA^{fMet}.

In archaebacteria and the cytoplasm of eukaryotes, protein synthesis is initiated with the initiator Met-tRNA (23). These initiator tRNAs contain an A:U base pair which is, in part, required for initiation factor (eIF) recognition of the tRNA (24). In contrast, eubacterial initiator tRNAs contain a C × A mismatch between nucleotides 1 and 72. The mismatch in the *E. coli* initiator tRNA is a key formylation determinant (4–6). Therefore, for initiation in the absence of formylation, it is possible that *P. aeruginosa* is using an initiator tRNA with functional properties comparable with those of eukaryotes. A search of the *P. aeruginosa* genome sequence data base identified two initiator tRNA genes encoding tRNAs that are almost identical. The sequences of the initiator tRNAs are homologous to the *E. coli* tRNA^{fMet} (Fig. 5). Moreover, the *P. aeruginosa* tRNAs contain a C × A mismatch between nucleotides 1 and 72. This feature was used by the *P. aeruginosa* MTF to recognize the initiator tRNA, since overproduction of the enzyme increased the initiator activity of a formylation-defective tRNA^{fMet} mutant with a base pair between nucleotides 1 and 72 (data not shown). These results suggest that *P. aeruginosa* is using the tRNA^{fMet} and not a unique initiator tRNA to initiate protein synthesis in the absence of formylation.

IF-2 is essential for initiation of protein synthesis in *E. coli* (25). The protein has a higher affinity for the fMet-tRNA^{fMet} than for the Met-tRNA^{fMet}, indicating that the formyl group is an IF-2 recognition element (9, 10). *In vitro* studies showed that IF-2 selects the fMet-tRNA^{fMet} at the initiation site of the preinitiation complex (9). Therefore, in *E. coli*, IF-2 is partly responsible for excluding the participation of the nonformylated Met-tRNA^{fMet} in initiation. This is consistent with the observation that in the MTF-deficient *E. coli* strain, initiation of protein synthesis is severely impaired. The eIF-2s of archaebacteria and eukaryotic cytoplasm are involved in selecting the initiator Met-tRNA for utilization in initiation of translation. A BLAST search of the *P. aeruginosa* genome data base identified sequences that were homologous to the *E. coli* IF-2 gene but none that were homologous for the eIF-2 gene. Therefore, it is conceivable that the *P. aeruginosa* IF-2 has dual substrate specificity and facilitates utilization of both Met-tRNA^{fMet} and fMet-tRNA^{fMet} in initiation or that an unidentified initiation factor, which only recognizes the Met-tRNA^{fMet} species, is involved.

We have shown that *P. aeruginosa* can carry out formylation-independent initiation of protein synthesis, using the Met-tRNA^{fMet}. This finding represents the first direct evidence that eubacteria have the ability to initiate translation with fMet-tRNA^{fMet} and Met-tRNA^{fMet}. While it is not known whether this phenomenon is unique to *P. aeruginosa*, it opens the possibility that other eubacteria may have the capacity to use both forms of the tRNA^{fMet} for initiation. The availability of various *fmt* sequences will facilitate investigation of this question in both Gram-positive and Gram-negative bacteria.

Acknowledgments—We thank Dr. J. S. Lam for the *P. aeruginosa* PAO1 strain and vectors. We also thank our colleagues, Drs. P. D. Josephy, F. J. Sharom, R. A. B. Keates, and A. R. Merrill, for their helpful suggestions during the preparation of this manuscript.

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J. Biol. Chem. 1999, 274:22143-22146.
doi: 10.1074/jbc.274.32.22143