Evidence that the Folate-Dependent Proteins YgfZ and MnmEG Have Opposing Effects on Growth and on Activity of the Iron-Sulfur Enzyme MiaB

Jeffrey C. Waller,^{a*} Kenneth W. Ellens,^a Ghulam Hasnain,^a Sophie Alvarez,^b James R. Rocca,^c and Andrew D. Hanson^a

Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, Florida, USA^a; Donald Danforth Plant Science Center, St. Louis, Missouri, USA^b; and AMRIS Facility, McKnight Brain Institute, University of Florida, Gainesville, Florida, USA^c

The folate-dependent protein YgfZ of *Escherichia coli* participates in the synthesis and repair of iron-sulfur (Fe-S) clusters; it belongs to a family of enzymes that use folate to capture formaldehyde units. Ablation of *ygfZ* is known to reduce growth, to increase sensitivity to oxidative stress, and to lower the activities of MiaB and other Fe-S enzymes. It has been reported that the growth phenotype can be suppressed by disrupting the tRNA modification gene *mnmE*. We first confirmed the latter observation using deletions in a simpler, more defined genetic background. We then showed that deleting *mnmE* substantially restores MiaB activity in *ygfZ* deletant cells and that overexpressing MnmE with its partner MnmG exacerbates the growth and MiaB activity phenotypes of the *ygfZ* deletant. MnmE, with MnmG, normally mediates a folate-dependent transfer of a formaldehyde unit to tRNA, and the MnmEG-mediated effects on the phenotypes of the $\Delta ygfZ$ mutant apparently require folate, as evidenced by the effect of eliminating all folates by deleting *folE*. The expression of YgfZ was unaffected by deleting *mnmE* or overexpressing MnmEG or by folate status. Since formaldehyde transfer is a potential link between MnmEG and YgfZ, we inactivated formaldehyde detoxification by deleting *frmA*. This deletion had little effect on growth or MiaB activity in the $\Delta ygfZ$ strain in the presence of formaldehyde, making it unlikely that formaldehyde alone connects the actions of MnmEG and YgfZ. A more plausible explanation is that MnmEG erroneously transfers a folate-bound formaldehyde unit to MiaB and that YgfZ reverses this.

Iron-sulfur (Fe-S) clusters are versatile cofactors that are typically liganded to cysteine residues in proteins (5, 19). Fe-S clusters are highly versatile; the proteins that contain them play diverse roles in electron transfer, enzyme catalysis, and transcriptional regulation (17). Although Fe-S clusters are simple structures, a complex machinery involving over 20 proteins is needed for their assembly, insertion into apoproteins, and maintenance (17). This machinery is incompletely understood (10, 19).

A newly discovered component of this machinery is the COG0354 protein family. COG0354 proteins are known to occur in all domains of life and to participate in the maturation of a subset of Fe-S proteins and in combating oxidative stress (12, 20, 28, 38). Null mutation of COG0354 causes growth defects in *Escherichia coli*, particularly under oxidative stress (20, 30, 37), as well as anemia in zebrafish embryos (29), a petite phenotype in yeast (12), and lethality in plants (39). The *E. coli* COG0354 protein, YgfZ, has been shown to help maintain the activity of the Fe-S enzyme MiaB, a tRNA modification enzyme that catalyzes the methylthiolation of *N*⁶-isopentenyladenosine (*i*⁶A) to 2-methylthio-*N*⁶-isopentenyladenosine (*ms*²*i*⁶A) (16, 30, 38). YgfZ was also demonstrated to maintain the activities of the Fe-S enzymes succinate dehydrogenase, dimethyl sulfoxide reductase, 6-phosphogluconate dehydratase, and fumarase (38). The biochemical function of COG0354 proteins remains unknown, but evidence from genetically manipulating *in vivo* folate contents indicates that *E. coli* YgfZ and its yeast counterpart Iba57p require a tetrahydrofolate (THF) species, almost certainly THF itself (11, 38) rather than a THF molecule with a 1-carbon substitution. Consistent with this evidence, YgfZ binds a stable model folate *in vitro*, and its three-dimensional (3-D) structure contains a predicted folate binding site (35, 38). Moreover, COG0354 proteins

are paralogous to the THF-dependent enzymes glycine cleavage T protein, sarcosine oxidase, and dimethylglycine oxidase (34, 35, 38). These three enzymes all use THF to accept a formaldehyde unit, specifically yielding 5,10-methylene-THF. It is thus reasonable to hypothesize that YgfZ accepts a formaldehyde unit using a THF molecule, as do its paralogs.

Ote et al. (30) reported that a growth phenotype of an *E. coli* *ygfZ* disruptant was partially suppressed by disrupting *mnmE* (*trmE*). MnmE and its partner MnmG (GidA) form the heterotrimer MnmEG, which uses 5,10-methylene-THF as the formaldehyde donor for a tRNA modification reaction (25). MnmEG thus mediates folate-dependent formaldehyde donation, whereas paralogs of YgfZ (and perhaps YgfZ itself) mediate folate-dependent formaldehyde removal. Given this possible reciprocity of action, the opposing effects of MnmEG and YgfZ, if confirmed, seemed likely to provide insights into the biochemical function of YgfZ. Accordingly, in this study, we first used deletants in a simple, well-defined genetic background to validate and extend the observations of Ote et al. (30) and then applied genetic, comparative genomic, and biochemical approaches to search for links between MnmEG and YgfZ.

Received 21 September 2011 Accepted 1 November 2011

Published ahead of print 11 November 2011

Address correspondence to Jeffrey C. Waller, jwaller@mta.ca.

* Present address: Department of Chemistry and Biochemistry, Mount Allison University, Sackville, New Brunswick, Canada.

Supplemental material for this article may be found at <http://jb.asm.org/>.

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doi:10.1128/JB.06226-11

MATERIALS AND METHODS

Bioinformatics. Prokaryote genomes were analyzed using the SEED database and its tools (31). Full results are available at <http://theseed.uchicago.edu/FIG/> in the YgfZ subsystem.

Bacterial strains, plasmids, and media. Strains, plasmids, and primers are listed in Tables S1 and S2 in the supplemental material. Deletions from the Keio collection (3) were transferred to *E. coli* K-12 MG1655 by P1 transduction (24); for double deletants, *kan* cassettes were removed by flippase-mediated recombination using the pCP20 plasmid (9). Deletions were verified by sequencing. Cells were grown at 37°C in antibiotic medium 3 (Difco), LB medium, MOPS (morpholinepropanesulfonic acid) minimal medium plus 0.2% (wt/vol) glucose (27), or M9 minimal medium plus 0.2% (wt/vol) glycerol as indicated below. Media were solidified with 15 g of agar per liter; ampicillin was added to 100 µg/ml. Gene expression was induced with 0.02% (wt/vol) L-arabinose. Where indicated below, plumbagin or formaldehyde was added to give final concentrations of 30 µM or 0.2 mM, respectively. Folate mutants were cultured as described previously (38).

Expression constructs. For the *mnmEG* construct, the *mnmE* open reading frame (ORF) was amplified from *E. coli* K-12 MG1655 genomic DNA with primers *mnmE*-Fwd and -Rev, digested with BspHI and XbaI, and ligated into similarly digested pBAD24 (14) to give pBAD24::*mnmE*. The *mnmG* ORF was amplified with primers *mnmG*-Fwd and -Rev, digested with NcoI and SalI, and ligated into similarly digested pET28b(+) (Novagen), yielding pET28b(+>::*mnmG*. An XbaI/SalI fragment (containing a 27-bp upstream spacer, the ribosome binding site, and *mnmG*) was excised from pET28b(+>::*mnmG* and ligated downstream of *mnmE* in similarly digested pBAD24::*mnmE* to give the bicistronic pBAD24::*mnmEG* construct. For pBAD24::*mnmG*, the NcoI- and SalI-digested *mnmG* amplicon was ligated into similarly digested pBAD24. All constructs were sequenced.

Immunoblot analysis. Cells were grown in antibiotic medium 3 to an optical density at 600 nm (OD₆₀₀) of 1.0, harvested by centrifugation (4,000 × *g* for 10 min at 4°C) and washed once in ice-cold phosphate-buffered saline. Proteins were extracted from cell pellets by lysis with a Mini-BeadBeater in 0.1 M Tris-HCl (pH 7.5), 0.2 M KCl, 3 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM benzamide, 1 mM phenylmethylsulfonyl fluoride, 5 mM ε-aminocaproic acid. Extracts were centrifuged (12,000 × *g* for 20 min at 4°C) to clarify them. Protein was estimated by dye binding (6), with bovine serum albumin as the standard. Electrophoresis and immunoblotting were performed as described previously (36). Antiserum was raised in rabbits (Cocalico Biologicals, Inc.) against hexahistidine-tagged, denatured YgfZ prepared as described previously (38) and was diluted 1:1,000.

MiaB activity analysis. Bulk nucleic acids were isolated from stationary-phase cells cultured in antibiotic medium 3 or MOPS medium plus glucose as described above and enriched for tRNA (4) before Nucleobond AXR 400 column purification (Machery-Nagel). Purified tRNA was then hydrolyzed and analyzed by liquid chromatography-tandem mass spectrometry as described previously (32). For statistical treatment, blocks were based on the replicates and were treated as random. Log-transformed data were analyzed by one-way analysis of variance (ANOVA) using Glimix procedures (SAS Institute, Inc., Cary, NC). Multiple comparisons were adjusted by Tukey-Kramer.

NMR analysis of *E. coli* cells incubated with [¹³C]formaldehyde. [¹³C]Formaldehyde (H¹³CHO; 99% atom percent) was from Cambridge Isotope Laboratories. Wild-type and *ΔfrmA* strains were grown in 100 ml of MOPS minimal medium to an OD₆₀₀ of 1.0. Culture samples (30 ml) were centrifuged (5,000 × *g* for 20 min at 4°C), and the pellets resuspended in 600 µl of 100 mM potassium phosphate buffer, pH 7.4, and kept on ice until analysis. Samples for nuclear magnetic resonance (NMR) contained 525 µl of cell suspension, 65 µl of D₂O, and 10 µl of 0.6 M H¹³CHO (final concentration 10 mM) (22). Directly detected 126-MHz ¹³C NMR spectra were obtained at 37°C on a Bruker Avance-500 instrument equipped with a 5-mm broadband observe (BBO) probe. Each data

FIG 1 The effects of deleting *mnmE* on the growth and MiaB phenotype of the *ΔygfZ* strain. (A) Growth of three independent clones of wild-type (WT), *ΔmnmE*, *ΔygfZ*, and *ΔmnmE ΔygfZ* strains on M9 medium plus 0.2% glycerol and 1 µM FeSO₄ after 4 days at 22°C or on LB medium with 30 µM plumbagin after 12 h at 37°C. (B) Liquid chromatography-mass spectrometry (LC-MS) quantification of i⁶A and ms²i⁶A and the ms²i⁶A/i⁶A ratio in tRNA of wild-type, *ΔmnmE*, *ΔygfZ*, and *ΔmnmE ΔygfZ* strains grown in antibiotic medium 3. Data are means and standard errors for three independent cultures.

set was acquired in 600 scans over a spectral width of 240 ppm using a 45° pulse, a 1-s acquisition time, and a 2-s relaxation delay. Composite-pulse ¹H decoupling was employed only during the acquisition and not during the delay, using the “zig” pulse program. The data, as free induction decay signals, were zero filled once and processed with 2-Hz exponential line broadening before Fourier transformation. Stacked spectral plots were prepared using Bruker’s XWinPlot software. Spectra were acquired at 30-min intervals.

RESULTS AND DISCUSSION

Deleting *mnmE* partially reverses *ΔygfZ* growth and MiaB activity defects. The phenotype exhibited by the *ΔygfZ* mutant in rich medium is moderately slowed growth; either oxidative stress (imposed with plumbagin) or growth in M9 minimal medium aggravates this defect (20, 30, 38). We therefore tested whether the reported suppression by *mnmE* disruption of the moderate *ygfZ* growth defect in rich medium (30) could be reproduced in oxidative stress conditions or in M9 medium, using full deletants in a well-defined (K-12 MG1655) background. This proved to be the case; deletion of *mnmE* in the *ΔygfZ* background largely reversed the severe *ΔygfZ* growth defect observed in plumbagin-containing or M9 medium (Fig. 1A). Deletion of *mnmE* alone had no effect (Fig. 1A).

To extend the analysis to the biochemical level, we measured the *in vivo* activity of MiaB, which is known to depend upon YgfZ

FIG 2 Exacerbation of the growth and MiaB phenotypes of the $\Delta ygfZ$ strain by overexpression of *mnmEG*. (A) Growth of three independent clones of wild-type (WT) and $\Delta ygfZ$ strains transformed with pBAD24 (pBAD) alone or pBAD24::mnmEG. The plate contained LB medium plus 0.02% L-arabinose. (B) LC-MS quantification of i⁶A and ms²i⁶A and the ms²i⁶A/i⁶A ratio in tRNA of the wild-type and $\Delta ygfZ$ strains transformed with pBAD24 alone or pBAD24::mnmEG. Strains were grown in antibiotic medium 3 plus 0.02% L-arabinose and 100 μ g/ml ampicillin. Data are means and standard errors for three independent cultures. For i⁶A and ms²i⁶A data, some error bars are too small to be visible. The i⁶A data for wild-type cells are shown in both normal ($\times 1$) and 100-fold-magnified ($\times 100$) formats.

(30, 38). The ratio of MiaB product to substrate, i.e., the ms²i⁶A/i⁶A ratio in tRNA, is a semiquantitative measure of MiaB activity (38). When cultured in antibiotic medium 3, wild-type *E. coli* typically has a ratio of 20 to 100, whereas that of $\Delta ygfZ$ mutants is < 2 (30, 38). As expected, wild-type cells showed a high ms²i⁶A/i⁶A ratio, as did $\Delta mnmE$ cells, and $\Delta ygfZ$ cells showed a low one (Fig. 1B). Relative to the results for the $\Delta ygfZ$ single mutant, the $\Delta mnmE \Delta ygfZ$ double mutant showed increased ms²i⁶A, decreased i⁶A, and restoration of the ms²i⁶A/i⁶A ratio to 28% of the wild-type level (Fig. 1B). Collectively, these data fit with the possibility that MnmEG damages MiaB, contributing to growth defects, and is in some way opposed by YgfZ.

Overexpressing MnmEG depresses growth and MiaB activity. Since *mnmE* deletion and, thus, loss of the MnmEG complex partially reversed the growth and MiaB activity phenotypes of the $\Delta ygfZ$ strain, we tested whether overexpression of MnmEG is detrimental. Overexpression of MnmEG in the $\Delta ygfZ$ background exacerbated the moderate growth defect in LB medium and also slightly reduced the growth of the wild type (Fig. 2A). Similar

FIG 3 Investigation of the folate dependence of the effect of MnmE on MiaB activity. LC-MS quantification of i⁶A and ms²i⁶A and the ms²i⁶A/i⁶A ratio in tRNA of the $\Delta folE$ and $\Delta folE \Delta mnmE$ strains grown in antibiotic medium 3. Data are means and standard errors for three independent cultures.

effects on MiaB activity were observed; overexpression of MnmEG reduced the already low ms²i⁶A/i⁶A ratio in the $\Delta ygfZ$ strain by a further 72% and caused a smaller (36%) but significant reduction in the much higher ratio in the wild-type strain (Fig. 2B). Expressing MnmE or MnmG alone did not affect the growth of the $\Delta ygfZ$ or wild-type strain (not shown). These results again fit with MnmEG-mediated damage to MiaB; they also indicate that this damage requires the enzymatically competent MnmEG complex and not merely its individual subunits.

Evidence that the detrimental effect of MnmEG requires folate. As already noted, MnmEG uses 5,10-methylene-THF in a tRNA modification reaction. We therefore investigated whether MnmEG-mediated damage to MiaB is also folate dependent. MiaB activity (ms²i⁶A/i⁶A ratio) was measured in a $\Delta folE$ mutant that lacks folates (38) and in the double $\Delta folE \Delta mnmE$ mutant. Because MiaB activity depends upon folate being available to YgfZ, it is reduced in $\Delta folE$ strains (38), but the remaining activity is sufficient to test the influence of MnmEG. Were the MnmEG-mediated reaction that damages MiaB folate independent, deleting *mnmE* in the $\Delta folE$ background should raise MiaB activity because the damage would be removed. Eliminating damage should enhance MiaB activity even though YgfZ is inactive for want of folate. Conversely, if the MnmEG damage reaction was strictly folate dependent, there should be no difference in MiaB activity between the $\Delta folE$ and $\Delta folE \Delta mnmE$ strains because damage would already be absent due to loss of folate. The latter result was observed: the ms²i⁶A/i⁶A ratios in the $\Delta folE$ and $\Delta folE \Delta mnmE$ mutants were the same (Fig. 3).

MnmEG expression and folate status do not affect YgfZ expression. The arguments in the three preceding sections assume that the MnmEG level and folate status do not affect YgfZ expression. Both assumptions were validated by immunoblot analysis: there was no effect on YgfZ expression from deleting *mnmE* or *mnmG* (see Fig. S1A in the supplemental material), overexpressing MnmEG (Fig. S1B), or deleting *folE* or from other genetic perturbations to the folate pool (Fig. S1C). YgfZ is induced by oxidative stress (7, 20), so the lack of effect of MnmEG overexpression (Fig. S1B) implies that the damage caused by MnmEG probably does not entail oxidative stress.

Examining formaldehyde-YgfZ-MiaB connections. Although 5,10-methylene-THF is normally made enzymatically, it can also form spontaneously from THF and formaldehyde from cellular reactions or the environment (1, 18, 21, 33). Formaldehyde also forms adducts with protein-bound thiol and amino groups spontaneously and readily (23) and, so, might in principle damage MiaB independently of MnmEG. We therefore sought

FIG 4 Effects of formaldehyde on the growth and MiaB phenotypes of single or double $\Delta frmA$ and $\Delta ygfZ$ mutant strains. Cells were grown in MOPS medium with 0.2% glucose with or without 0.2 mM formaldehyde for 48 h. At earlier times (e.g., 36 h) the double mutant showed marginally less growth than other strains. (A) Growth of three independent clones of wild-type (WT), $\Delta frmA$, and $\Delta ygfZ$ strains. The formaldehyde detoxification pathway is shown at the top. GSH, glutathione; GS-CH₂OH, S-hydroxymethylglutathione; GS-CHO, S-formylglutathione. (B) LC-MS quantification of $i^6\text{A}$ and $ms^2i^6\text{A}$ and the $ms^2i^6\text{A}/i^6\text{A}$ ratio in tRNA of wild-type, $\Delta frmA$, $\Delta ygfZ$, and $\Delta frmA \Delta ygfZ$ strains. Data are means and standard errors for three independent cultures. Means not designated with the same letter were significantly different at a *P* value of 0.05 (see Materials and Methods). Note that these experiments used MOPS minimal medium to prevent formaldehyde titration by adduct formation with medium components; the $ms^2i^6\text{A}/i^6\text{A}$ ratios are thus not directly comparable to those in other figures, where rich antibiotic medium A was used.

links between formaldehyde, YgfZ, and MiaB using comparative genomic and experimental approaches.

Formaldehyde is metabolized mainly via its spontaneous adduct with glutathione (S-hydroxymethylglutathione), which is oxidized to S-formylglutathione by FrmA; S-formylglutathione is then hydrolyzed by FrmB and YeiG, giving formate (Fig. 4A) (13). A survey of 858 bacterial genomes in the SEED database (31) revealed no tendency for *frmA* or other formaldehyde metabolism genes to cluster on the chromosome with *ygfZ*. Nor were *frmA* and *ygfZ* codistributed: *ygfZ* occurred without *frmA* in 197 genomes and *frmA* occurred without *ygfZ* in 2 genomes, 303 genomes had both genes, and 353 had neither. Comparative genomics thus detects no link between formaldehyde metabolism genes and YgfZ. However, the methyltrophic bacterium *Methylophilales* bacterium HTCC2181 has a COG0354 protein family member in an operon with proteins involved in methanol dehydrogenase activity, a reaction known to generate formaldehyde (15).

In our experimental approach, we first tested single and double *ygfZ* and *frmA* deletants for sensitivity to supplied formaldehyde, selecting a concentration (0.2 mM) shown in pilot tests to slightly inhibit the growth of wild-type cells in minimal medium. (Minimal medium was used to avoid formaldehyde adduct formation with the organic constituents of rich medium.) No deletant strain

was substantially more sensitive to formaldehyde than the wild type (Fig. 4A). We then analyzed MiaB activities in the presence and absence of formaldehyde (Fig. 4B). As expected, the $ms^2i^6\text{A}/i^6\text{A}$ ratio was significantly depressed in the $\Delta ygfZ$ strain, although the magnitude differed from that in Fig. 1B due to the use of minimal medium. Deleting *frmA* had little effect alone or combined with the *ygfZ* deletion, whether or not formaldehyde was supplied. These data indicate that formaldehyde itself, as opposed to its folate-bound form 5,10-methylene-THF, does not cause the damage to MiaB that YgfZ opposes.

The above-described inference rests on the assumptions that supplied formaldehyde reaches the cytosol, rather than being intercepted by adduct formation in the cell wall or periplasm, and that deleting *frmA* raises cytosolic formaldehyde levels. To test both assumptions, we gave [¹³C]formaldehyde to wild-type or $\Delta frmA$ cells and followed its fate by NMR. In wild-type cells, [¹³C]formaldehyde, observed as its hydrate H₂¹³C(OH)₂ and its glutathione adduct, was progressively metabolized to [¹³C]formate (see Fig. S2 in the supplemental material). In the $\Delta frmA$ strain, however, little [¹³C]formate was formed and the hydrate remained predominant (Fig. S2). These data thus validate the two assumptions, although it should be noted that, in order to obtain strong NMR signals (22), a higher formaldehyde level was used

than in the experiments on growth and MiaB activity (10 mM versus 0.2 mM).

Conclusions. Biochemical roles for YgfZ have been proposed in the past but were unsupported by experimental data (30, 35). Here, we present genetic and biochemical evidence that the phenotypes of $\Delta ygfZ$ strains are due, in part, to a folate-dependent reaction catalyzed by MnmEG that damages MiaB and that the folate-dependent action of YgfZ counters this damage. Our data also indicate that MnmEG is not the sole source of MiaB damage; were it so, deleting *mnmE* in the $\Delta folE$ strain would have fully restored MiaB activity (Fig. 1B) and MiaB activity in the $\Delta folE$ strain would have been higher than it was (Fig. 3). Moreover, YgfZ occurs in many genomes that lack MnmEG (e.g., *Actinobacteria*), implying that MiaB is subject to additional types of damage.

The *miaB* deletant has relatively subtle growth phenotypes (28), whereas overexpressing *mnmEG* in the $\Delta ygfZ$ background causes quite severe growth and MiaB activity phenotypes. It would consequently seem unlikely that MiaB is the only enzyme affected by the detrimental action of MnmEG, just as it is not the only one benefited by YgfZ. It is therefore reasonable to speculate that, in relation to MnmEG-mediated damage, MiaB is in effect a proxy for other enzymes, as it is in relation to Fe-S enzyme activity loss when *ygfZ* is deleted (38).

Many cases are known where macro- or micromolecules are damaged by enzymatic mistakes or spontaneous chemical reactions and then enzymatically repaired (8, 37). The opposing effects of MnmEG and YgfZ could reflect a system of this kind. Given that the MnmEG complex normally transfers a formaldehyde unit from folate to a tRNA (25), that close relatives of RNA modification enzymes modify proteins instead (2, 26), and that proteins form formaldehyde adducts (23), it is conceivable that MnmEG occasionally and by mistake transfers a formaldehyde unit to a sensitive residue in MiaB and, perhaps, to such residues in other proteins. YgfZ might strip this unit off and transfer it to THF. Evidence for such a system might in principle be obtained from a proteomics search for modifications to MiaB that appear in the $\Delta ygfZ$ mutant. However, as formaldehyde adduct formation is reversible (18, 23), such a search would not be straightforward.

ACKNOWLEDGMENTS

This work was supported in part by National Science Foundation grant number MCB-0839926 and by an endowment from the C.V. Griffin Sr. Foundation. We gratefully acknowledge the National Science Foundation through the National High Magnetic Field Laboratory, which supported our NMR studies, in part, at the Advanced Magnetic Resonance Imaging and Spectroscopy (AMRIS) Facility in the McKnight Brain Institute of the University of Florida.

We thank S. M. Beverley, Arthur S. Edison, and V. de Crécy-Lagard for advice. Statistical analysis was provided by Dongyan Wang of the IFAS Statistics Department at the University of Florida.

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