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Ribosomal protein L9 is a component of all eubacterial ribosomes, yet deletion strains display only subtle growth defects. Although L9 has been implicated in helping ribosomes maintain translation reading frame and in regulating translation bypass, no portion of the ribosome-bound protein seems capable of contacting either the peptidyltransferase center or the decoding center, so it is a mystery how L9 can influence these important processes. To reveal the physiological roles of L9 that have maintained it in evolution, we identified mutants of *Escherichia coli* that depend on L9 for fitness. In this report, we describe a class of L9-dependent mutants in the ribosome biogenesis GTPase Der (EngA/YphC). Purified mutant proteins were severely compromised in their GTPase activities, despite the fact that the mutations are not present in GTP hydrolysis sites. Moreover, although L9 and YihI complemented the slow-growth *der* phenotypes, neither factor could rescue the GTPase activities *in vitro*. Complementation studies revealed that the N-terminal domain of L9 is necessary and sufficient to improve the fitness of these Der mutants, suggesting that this domain may help stabilize compromised ribosomes that accumulate when Der is defective. Finally, we employed a targeted degradation system to rapidly deplete L9 from a highly compromised *der* mutant strain and show that the L9-dependent phenotype coincides with a cell division defect.

Ribosomal proteins are a curious class of translation factors in that most of them do not appear to participate directly in protein synthesis (1). Although the roles of some ribosomal proteins may be to maintain the architecture of the ribosome active centers, many typically possess regions of high conservation in areas that do not contact other ribosomal proteins or rRNA. There is mounting evidence that the conserved motifs in some ribosomal proteins are used either to regulate translation or to connect ribosomes to other important cellular processes (2–5). Interestingly, some very highly conserved ribosomal proteins can be deleted from bacteria without inducing appreciable growth phenotypes, which obfuscates determination of their molecular functions (2, 6). The bacterium-specific ribosomal protein L9 is an example of this nonessential class: it possesses a conserved secondary and tertiary architecture and contains several invariant amino acids, yet deletion strains grow well (7–9). From the perspective that all highly conserved factors serve as windows to important cellular processes, we reasoned that a deeper understanding of L9 would help connect this enigmatic ribosomal protein to basic bacterial physiology.

Ribosomal protein L9 was initially characterized during *in vitro* ribosome assembly studies in the early 1980s (10, 11). From those studies, it was established that L9 is a primary ribosome binding protein in that it does not require other proteins to engage the 23S RNA. A functional role for L9 in reading frame maintenance came from a genetic screen for *Escherichia coli* mutants that increased translation through a partially defective bacteriophage T4 gene *60* bypass region (12). The bypass event during gene *60* translation is remarkable in that the ribosome recognizes signals in the nascent peptide and mRNA that promote a 50-nucleotide "hop" before reengaging the same mRNA to complete the synthesis of the encoded protein (13). The *hop-1* mutation was recovered and identified as a Ser93Phe alteration in a highly conserved patch on the C terminus of L9. Subsequent studies demonstrated that L9 also influences frameshifting at codon repeats and stop codons (12, 14–16). More recently, L9 deletion strains were shown to read through stop codons more frequently (out of frame) and encounter the 3′ ends of their engaged mRNAs, which then invokes ribosome rescue systems (8). Thus, at least one role for L9 is in maintaining translation fidelity, but nothing is known about the mechanism for this activity.

Structurally, L9 is odd in that it projects from the surface of the large subunit near the base of the L1 stalk. L9's architecture is very highly conserved and is comprised of a globular N domain that docks with the 23S RNA, a long alpha helix of fixed length, and a globular C domain displayed away from the surface in crystal structures (Fig. 1) (17–19). Although the positioning of L9 in crystal structures implies a rigid conformation, chemical footprinting and cross-linking experiments suggest that L9 is dynamic and may engage portions of the L1 stalk RNA and also surrounding regions of the large subunit (20, 21). In support of this idea, structural biologists recently demonstrated that L9 might be inadvertently stabilized in ribosome crystals through interribosomal contacts. Indeed, ribosomes missing L9 can enter alternative crystal forms, a feature that allowed, for the first time, resolution of the GTPase-activating center (9, 19, 22).

Not only is the architecture of L9 conserved, but also there are collections of invariant amino acids in both the N and C domains. Considering the expansive evolutionary history of L9 in eubacteria (it is conserved in all bacterial phyla), it would seem that L9 plays a critical role in cell physiology that is frequently selected in nature. However, in tested cases, L9 deletion strains appear

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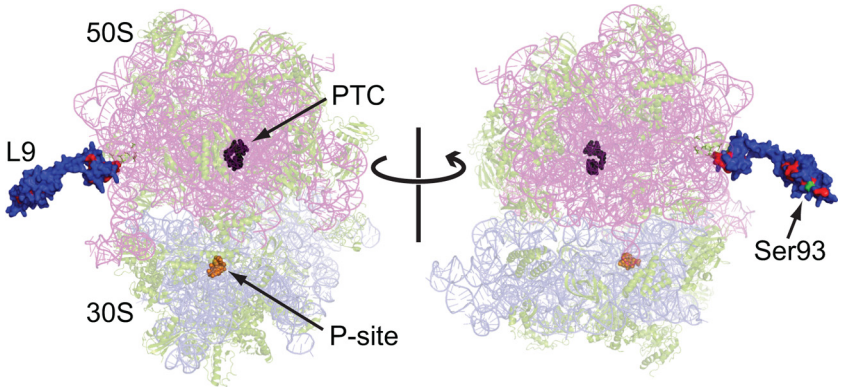

FIG 1 L9 on the ribosome. A rendering of a crystal structure of the *E. coli* ribosome with L9 conservation is shown in two views (Protein Data Bank files 2i2t and 2i2p). The 50S and 30S subunits are indicated with 23S rRNA in pink, and 16S rRNA is in slate. The locations of the peptidyltransferase center (*PTC*) and the P-site are highlighted. L9 is surface rendered in blue, with invariant amino acids in red. The *hop-1* residue that affects translation bypass, Ser93, is colored green.

healthy. While it can be argued that a small fitness advantage is sufficient for evolutionary conservation, the other domains of life do not have L9 (aside from bacterium-like organelles).

The conservation of specific residues in L9 suggests that it interacts with other factors that are also very highly conserved. We are interested in identifying such factors, not only to develop a mechanistic understanding of L9's role in translation fidelity but also to potentially reveal new biochemistries. To move in this direction, we screened a chemically mutated *E. coli* library for mutants that depend on L9, hoping we could recover mutations in essential factors that would point to the functions of L9. Here, we describe strains with mutations in the essential ribosome biogenesis GTPase Der (EngA/YphC) that grow better with L9 than without it (23, 24). We show that the L9-dependent Der mutants are severely compromised for GTPase activity, which was previously shown to be critical for Der's essential function (23, 25, 26). Purified L9 does not rescue the GTPase defects, nor does it alter the stimulatory activity of Der's GAP-like factor YihI, so the fitness afforded by L9 seems indirect. We put forward a preliminary hypothesis that the ribosome-binding domain of L9 may help stabilize structurally compromised large subunits synthesized when Der activity is limiting.

MATERIALS AND METHODS

Strains and plasmids. Strain TB28 (MG1655, Δ*lacIZYA*) was used as wild-type (WT) *E. coli* for this study (27). The gene encoding L9 (*rplI*) was deleted or modified by recombineering in strain SM1405 (X90, Δ*clpX* Δ*clpA* harboring plasmid pSIM5) using selection for either a promoterless Kan^r open reading frame (ORF) or promoter-containing Tet^r or Cat^r genes (28). Mutants of *rplI* were P1 transduced into TB28 and the modifications verified by diagnostic PCR and DNA sequencing (29, 30). For complementation, Der variants, full-length L9 (1 to 149), L9$^{1\text{-}149}$-FLAG-His$_6$, L9$^{1\text{-}53}$-FLAG-His$_6$, L9$^{65\text{-}149}$-FLAG-His$_6$, and YihI-FLAG-His$_6$ were expressed from derivatives of the pTrc99a plasmid (31). Der variants were overexpressed for purification from pET-3a (Novagen) (32). The construction of the unstable reporter plasmid used for the screen is described in the supplemental material.

Chemical mutagenesis and library screening. The screening strain AN226 (TB28, Δ*rplI*::*tet* harboring pRC-L9) was mutated using *N*-ethyl-*N*-nitrosourea (ENU; Sigma number N3385) using a published protocol as a guide (33). An overnight culture of AN226 was diluted (1/50) in 1 ml of A-0 medium with 0.2% glycerol, 0.5 mM isopropyl-β-D-thiogalactopyranoside [IPTG], and 75 μg/ml of ampicillin (34). At early exponential phase, 26 mM ENU (stock prepared in 0.1% acetic acid and 23% dimethyl sulfoxide [DMSO]) was added to the culture. A parallel control culture received only the ENU diluent. After 20 min at room temperature, the cells were recovered in 1 ml of LB medium containing 0.5 mM IPTG and 28 mM 2-mercaptoethanol (to inactivate the mutagen) for 2 h at room temperature. The culture that received ENU exhibited ~95% loss in viability compared to the mock. Dilutions of the library were plated on LB agar (with 40 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal], 0.2% glycerol, and 0.5 mM IPTG) and screened at ~250 colonies per 90-mm plate.

These mutants were cured of pRC-L9, and mutations causing the phenotype were mapped using transposon-based P1 transduction marker rescue (see the supplemental material). The mutations were then identified by sequencing the mapped locus. Three mutants had changes to *der*. All sequences were analyzed using the *E. coli* K-12 MG1655 genome as the wild type (GenBank accession number U00096.2) (35).

Protein expression and purification. L9-FLAG-His$_6$ was purified under denaturing conditions, refolded on an Ni^{2+} column by desalting, and then eluted under native conditions (see the supplemental material). YihI-FLAG-His$_6$ was purified similarly but under native conditions. L9 and YihI were further purified by hydroxyapatite chromatography. Purified proteins were exchanged into buffer A (20 mM Tris-HCl, 25 mM NaCl, 0.05% Tween 20, 5% glycerol, and 5 mM 2-mercaptoethanol [pH 8.0]) prior to storage at −80°C. The concentrations were determined by UV absorbance ($\varepsilon_{\text{L9 in GuHCl}} = 1{,}280\ M^{-1}\ cm^{-1}$ and $\varepsilon_{\text{YihI in GuHCl}} = 6{,}890\ M^{-1}\ cm^{-1}$) (36).

We were unable to obtain ample soluble Der with either N- or C-terminal epitope tags and strains with C-terminally tagged chromosomal *der* were very sick, suggesting a defective enzyme. Therefore, wild-type and mutant versions of untagged Der were overexpressed using a T7 expression system (pET-3a; Novagen) and purified conventionally (see the supplemental material). Briefly, overexpressed protein was purified from cleared lysates under native conditions using a combination of anion exchange, hydroxyapatite binding, and ammonium sulfate precipitations. Purified Der contained a contaminant that exhibited the absorbance profile of nucleic acid (likely GDP as has been reported previously) (37–39), which prevented quantification using UV absorbance. Therefore, the concentration of Der was measured using Bradford assays with bovine serum albumin (BSA) as a standard (Bio-Rad).

GTPase assays. Der's GTPase activity was measured using a regenerative coupled assay (40). A 20× assay mix (20 mM NADH, 150 mM phosphoenolpyruvate, and ~10 U/ml of pyruvate kinase/lactate dehydrogenase mixture [Sigma; number P0294]) was prepared in assay buffer (20 mM Tris-HCl, 100 mM KCl, 0.05% Tween 20, 5% glycerol, 5 mM 2-mercaptoethanol [pH 8.0]) and frozen in aliquots at −80°C.

It is established that Der's GTPase rate is increased at high concentrations of potassium (24, 39). In preliminary experiments, we determined that by increasing KCl or KPO_4, the rate of GTP hydrolysis could be accelerated to the point that stimulatory effects of YihI were no longer measureable (see the supplemental material). Therefore, our assay buffer was formulated to set the basal rate of wild-type Der at ~50% the YihI-stimulated rate so that stimulatory effects could be readily observed. Although the T57I and E271K mutants were also stimulated by potassium, the relative turnover differences were not affected. Assay mixes (2×) were prepared in assay buffer supplemented with GTP and a 2 mM $MgCl_2$ excess over the GTP concentration. Thirty microliters of the 2× assay mixture was combined with 30 μl of 2× enzyme (diluted in assay buffer). Fifty microliters of the reaction mixture was then transferred to a 96-well plate, and the loss of absorbance of NADH was monitored at 340 nm at 1-min intervals. The slopes of straight lines fitted to the raw data were converted to GTPase rates using the NADH extinction coefficient, and the background rates of controls lacking GTPase were subtracted (40). Doping of GDP into pilot reactions established that the regeneration system was capable of converting >150 μM GDP to GTP min^{-1}. Reaction rates were typically linear over several hours. The K_m and V_{max} values were determined at various GTP concentrations by fitting to the Michaelis-Menten equation using Prism 6 (GraphPad software). The affinity of YihI for Der was determined by converting the stimulation data to fractional occupancy and fitting to the law of mass action to determine K_d (dissociation constant) (see the supplemental material).

Conditional degradation. The conditional degradation system has been described elsewhere (29). Briefly, the endogenous target gene was modified to encode a C-terminal peptide tag that is recognized by the processive unfoldase/protease ClpXP. The expression of ClpXP was then regulated from a plasmid. Strains were maintained in glucose to repress expression of ClpXP and then switched to medium containing arabinose to induce the protease and degrade the target. For this study, recombineering was used to replace wild-type *rplI* with *rplI-deg* or *rplI-cont* using a downstream antibiotic marker for selection (*rplI* is the last gene in the S6 operon) (28). P1 transduction was used to move tagged versions of *rplI* into a Δ*clpX* strain with WT or mutant *der*. The strains were then transformed with a pBR-ClpXP plasmid library with randomized Shine-Dalgarno sequences to select candidate plasmids that allowed optimum expression of ClpXP for L9 degradation (29). Transformants were tested for L9 degradation in the first ~30 to 40 min of induction using Western blotting. For degradation experiments, a rich defined morpholinepropanesulfonic acid (MOPS)-buffered medium was used to better control catabolite responses (Teknova) (41).

Mutant *derT57I* strains carrying tagged *rplI* and pBR-ClpXP were diluted from an overnight culture (1/100) into medium containing 100 μg/ml of ampicillin and 0.2% glycerol, and 100 μl was grown with continuous shaking in a 96-well plate at 37°C (Biotek Synergy MX). At early exponential phase, either 0.2% arabinose or 0.2% glucose was added. After ~40 to 50 min of induction, samples of the cultures were normalized for their absorbance at 600 nm for Western analysis, and a separate aliquot was diluted (1/10) into a new well containing either glucose or arabinose medium. When the cultures reached a density nearing the end of exponential phase, the sampling and dilution were repeated.

Microscopy. Cells from control and L9-depleted cells were imaged using differential interference contrasting (DIC) from phosphate-buffered saline (PBS)-washed samples of liquid cultures. Cells were heat fixed onto slides and covered with mounting medium (ProLong gold; Invitrogen) prior to imaging (Zeiss Axiocam MRc5, DIC III). Cell lengths were measured using software from the microscope manufacturer (Axiovision, version 4.5), analyzed in Excel (Microsoft), and plotted using Prism (GraphPad).

RESULTS

Mutations in *der* cause a dependence on L9. To reveal pathways influenced by L9 in *E. coli*, we carried out a synthetic-lethality screen for mutations in other genes that compromise cell health in L9's absence (27). We first deleted the chromosomal gene encoding L9 (*rplI*) by replacing it with a tetracycline resistance gene. Consistent with previous reports that L9 is nonessential, the Δ*rplI* strain formed colonies that were indistinguishable from *rplI*$^+$ cells and exhibited only a slight reduction in yield in liquid cultures (7–9). We then placed a clone of the L9 open reading frame (ORF) on an unstable reporter plasmid under the control of a controllable promoter (P_{trc}). The resulting strain was chemically mutated to generate a library and screened for colonies that retained the reporter plasmid (synthetic lethality, indicating that L9 improved their fitness). Potential L9-dependent mutants (exhibiting a solid blue colony phenotype) were recovered at approximately 1 in 20,000 colonies.

Three *rplI*-dependent mutants mapped to a common locus, and DNA sequencing revealed that each had a point mutation in the ORF of the *der* gene (also called *engA* and *yphC*) (24, 42). The mutated *der* genes encode DerT57I and DerE271K (DerT57I was recovered and mapped twice independently). The T57I and E271K mutations alter very highly conserved residues in each of Der's two GTPase domains (G domains) (23, 37). T57 is within the G3 motif of G domain 1, and E271 is within the switch II motif of G domain 2. When cured of the support plasmid that supplies L9, the *derT57I* mutant was sicker than the *derE271K* mutant, but each still formed colonies (Fig. 2A).

To verify that *rplI* indeed improved the fitness of the recovered *der* mutants, we used phage transductions to replace the *rplI* locus in the mutants with either another L9 null mutation (Δ*rplI*::*cat*, as a control) or the wild-type genotype (*rplI-cat*). Replacing the existing Δ*rplI*::*tet* mutation with the Δ*rplI*::*cat* mutation did not rescue the growth defects. In contrast, restoring *rplI* in the chromosome improved growth to a level that was intermediate between the recovered *rplI der* mutants and an *rplI*$^+$ *der*$^+$ strain (Fig. 2B). Thus, the fitness of these *der* mutants is increased when the cells have L9, but the mutant *der* genes cause growth reductions, suggesting that they remain partially defective in the presence of L9.

The N domain of L9 complements *derT57I*. L9 contains highly conserved amino acids in both its N and C domains. To determine if either domain could suppress the small-colony phenotype independently of the other, we expressed them from plasmids in the *derT57I* strain because of its easily scorable phenotype (L9 residues 1 to 53 and 65 to 149). Full-length and N domain constructs improved the growth to comparable extents on plates and in liquid cultures (~60% of the growth rate of the *der*$^+$ strain). The C domain did not complement and grew similarly to the *derT57I* strain with a mock plasmid (~30% of the growth rate of the *der*$^+$ strain [Fig. 2C and data not shown]). We established that the C domain construct expressed protein of the predicted size from this construct in a separate experiment (data not shown). Therefore, the N domain of L9 is necessary and sufficient for complementation of the *derT57I* allele.

The *derT57I* and *derE271K* mutants are partially functional and recessive. We cloned the *der*$^+$, *derT57I*, and *derE271K* ORFs onto plasmids and introduced them into Δ*rplI*, *der*$^+$, and *der* mutant strains. A mock plasmid lacking *der* was used as a control. Because homologous recombination was active in these strains and capable of replacing the mutant *der* loci, we plated dilutions of freshly transformed cells to evaluate colony fitness without substantial outgrowth. This procedure also reduced the accumulation of second-site suppressors (described be-

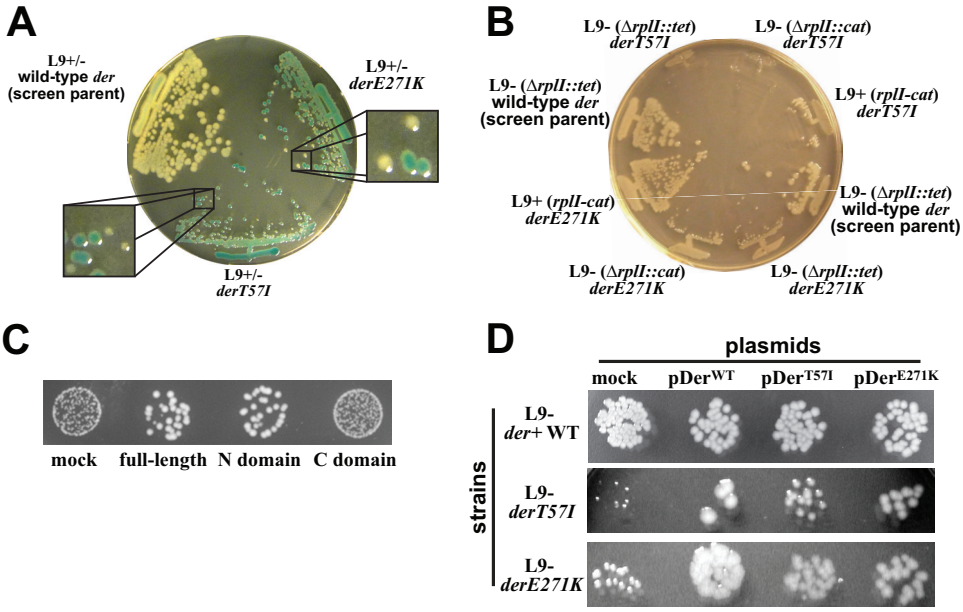

FIG 2 L9 improves the health of *der* mutants. A synthetic-lethality screen revealed mutants that grow better with an unstable reporter plasmid expressing L9. (A) Comparison of the parental screening strain to two recovered *der* mutants on an X-Gal indicator plate. The parental cells did not require L9 and turned white during colony development from plasmid loss. Cells that grew better with L9 maintained a blue color in the colony because plasmid-containing cells were more fit. The *derT57I* strain was sicker in the absence of L9 than the *derE271K* strain, evidenced by the relative colony sizes without the reporter plasmid. (B) Strains cured of the reporter plasmid were transduced to replace the *rplI* locus in the chromosome. A control transduction replaced the original Δ*rplI*::*tet* mutation with the Δ*rplI*::*cat* mutation and did not improve growth. Restoring rplI (*rplI-cat*) improved the health of both *der* mutants but not to the level of the parental cells with wild-type *der*. (C) Complementation of the Δ*rplI derT57I* mutant with a mock plasmid or plasmids encoding $L9^{1-149}$-FLAG-His_6 (full length), $L9^{1-53}$-FLAG-His_6 (N domain), or $L9^{65-149}$-FLAG-His_6 (C domain). The N domain alone complemented the small-colony phenotype as well as full-length L9. (D) Transformation with plasmids that express wild-type or mutant Der to test for *trans*-complementation of the chromosomal *der* alleles. Wild-type Der restored full health to each mutant (second column). Overexpression of either the T57I or E271K mutant improved the health of each mutant but did not sicken cells with wild-type *der* in the chromosome. Therefore, each der mutant is partially functional and recessive.

low). Introducing plasmid-borne der^+ into the *derT57I* and *derE271K* strains fully restored colony and liquid culture growth (Fig. 2D, second column, and data not shown). This finding indicates that the slow-growth phenotypes were caused solely by the mutations in *der*. Plasmid-borne versions of either *derT57I* or *derE271K* partially restored the growth of strains with the same alleles and also of the other mutant (Fig. 2D, second and third columns). Moreover, these plasmids did not sicken cells with chromosomal der^+. Therefore, the recovered *der* mutants are recessive, and they encode partially active Der variants that support growth better when more is expressed.

Suppressor mutations arise frequently in the *derT57I* background. Our efforts to transduce the mutant genes to other strains were impeded by the weak screenable phenotype of *derE271K* and a rampant accumulation of escape mutants of *derT57I* that grew well and lost their dependence on L9. The observed frequency of escape in overnight cultures (10^{-2} to 10^{-5}) of *derT57I* was too high to be accounted for by same-site reversion (expected at $\sim 10^{-9}$). Therefore, to determine if the escape mutations were intra- or extragenic, we sequenced the *der* genes from four *derT57I* fast-growing escape mutants. Each retained the original T57I mutation but contained an additional mutation in the same GTPase domain near the T57I position (Fig. 3). This finding reinforces the conclusion that the T57I mutation in Der is solely responsible for the slow-growth phenotype and the dependence on L9. Also, unsuppressed *derT57I* strains are not able to be reliably cultured for biochemical studies.

The T57I and E271K mutations impair the GTPase activity of Der. Prior studies indicated that GTP hydrolysis by each of the two GTPase domains is required for *E. coli* Der's essential function and that they act cooperatively (23, 37). We discovered that *der* encoding a C-terminal FLAG-His_6 tag was unable to functionally replace wild-type *der* in the chromosome. Very sick strains with these tags spawned fast-growing escape mutants with frameshift

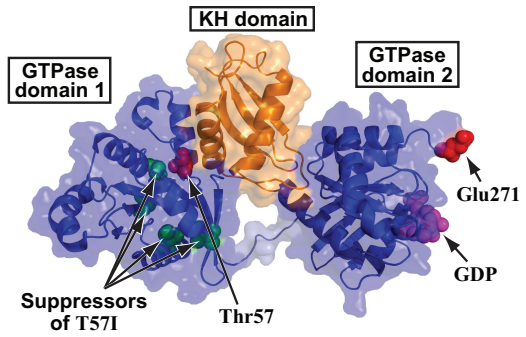

FIG 3 Locations of the L9-dependent Der mutants and T57I mutant suppressors. Shown is a rendering of Der from *Thermotoga maritima* (Protein Data Bank code 1MKY) showing the locations of T57 and E271 and the relative positions of four *E. coli* T57I mutant suppressors (A45V, R109G, T113A, and V158G, in green). The GDP bound in G domain 2 is pink. In this conformation of Der, the T57I mutation lies at the interface between G domain 1 and the KH domain. The E271K mutation is in switch II of G domain 2.

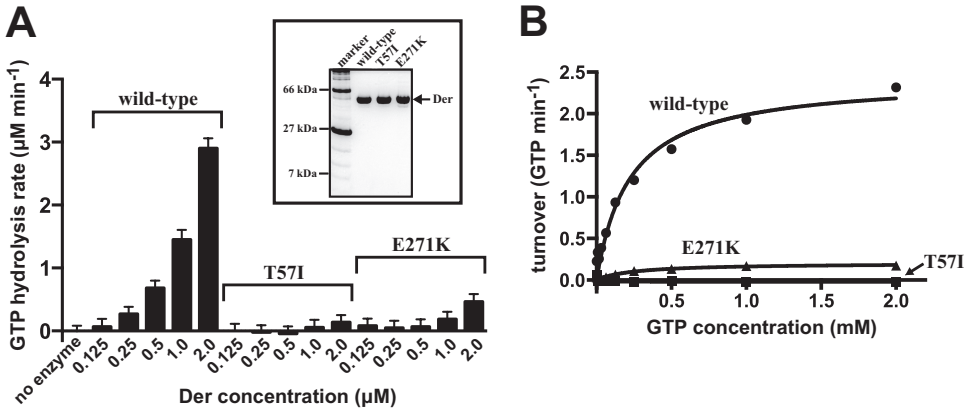

FIG 4 DerT57I and E271K are compromised in their GTPase activities. (A) Various concentrations of wild-type, T57I, and E271K Der proteins were evaluated in a regenerative GTPase assay using a GTP concentration that nearly saturated the wild type (1 mM). (Inset) Coomassie-stained SDS-PAGE of the purified Der proteins. Increasing the wild-type Der concentration increased the observed GTP hydrolysis at each concentration tested. The T57I and E271K mutants displayed a measureable hydrolysis rate above background only at high concentrations (~2 μM for the T57I mutant and ~1 μM for the E271K mutant). The error bars are the standard deviations from three measurements. (B) Michaelis-Menten kinetic analysis of each protein at various GTP concentrations (0.008 to 2 mM). The wild type was assayed at 0.5 μM and the T57I and E271K mutants were each assayed at 2 μM, and then the rates were converted to turnover rate per enzyme. The K_m and V_{max} values for the wild type were 0.22 ± 0.04 mM and 2.42 ± 0.16 min^{-1}, respectively, and these values for the E171K mutant were 0.25 ± 0.06 mM and 0.20 ± 0.01 min^{-1}, respectively. The rate of GTP hydrolysis by T57I was too low for fitting.

mutations in the 5′ end of *der* that prevented expression of the tagged enzyme. To avoid the possibility of aberrant behaviors stemming from tags on Der, the wild-type, T57I, and E271K versions were purified as untagged proteins for *in vitro* characterization.

The reported apparent K_m of *E. coli* Der for GTP is ~140 μM (23). Therefore, we preliminarily measured the basal GTP hydrolysis rates for each enzyme at 1 mM substrate (near saturating) using 0.125 to 2 μM enzyme. Under these conditions, we observed a dose-dependent increase in the GTP hydrolysis rate for the wild-type enzyme and a turnover of ~1.5 min^{-1}, which is consistent with the reported basal GTPase activity of Der (Fig. 4A). Both mutants were severely compromised in their GTPase activities, with the E271K mutant possessing a higher turnover rate than the T57I mutant (~0.30 and ~0.02 min^{-1}, respectively). Thus, the severity of the GTPase defects mirrored the severity of the growth phenotypes. Moreover, each mutation inhibited the GTPase activity of both GTPase domains, which supports a proposed highly cooperative hydrolysis mechanism for Der (23).

To determine if the observed rate defects were from a loss in affinity for GTP or from a catalytic defect, we measured the hydrolysis rates under various GTP concentrations to obtain K_m and V_{max} values (Fig. 4B). Under these conditions, wild-type Der exhibited an apparent K_m of ~0.22 mM and a V_{max} of 2.42 min^{-1}. We were unable to obtain these kinetic parameters for the T57I mutant because the hydrolysis rates were too low for fitting, but the E271K mutant exhibited an apparent K_m of ~0.25 mM and a V_{max} of 0.2 min^{-1}. Thus, this mutant was not compromised in its ability to bind GTP, and the observed low rate may stem from a reduction in the mechanical cycling of the enzyme because the E271 residue is not in contact with the GTPase center.

Additional YihI partially complements the *der* mutants. A recent study identified the highly conserved, nonessential protein YihI as a factor that stimulates Der (43). In particular, YihI was reported to increase Der's GTPase V_{max} by ~50% and decrease its K_m by ~50%. DNA sequencing revealed that *yihI* was wild type in each of our *der* mutant strains. To determine if YihI influenced the fitness of the L9-dependent *der* mutants, we cloned its ORF onto a multicopy plasmid under the control of the P_{trc} promoter and introduced it into the *der* mutants. We and others observed that high levels of YihI severely inhibited growth (data not shown) (43); therefore, we tested for complementation under noninducing conditions, wherein leaky expression from the plasmid would moderately overexpress YihI. Under these conditions, the additional YihI complemented both the colony and liquid culture growth of the *derT57I* mutant as well as L9 did (~60% recovery of growth rate), but neither protein complemented as well as wild-type Der (full restoration) (Fig. 5A and data not shown). Providing additional YihI only subtly improved *derE271K* mutant growth. These findings suggest that YihI helps these mutants deal with their defective Der; however, we show below that this factor does not restore their GTPase activities.

YihI stimulation is potassium sensitive. To directly evaluate the influence of YihI on the Der mutants, we purified YihI so we could monitor its stimulatory effect in GTPase assays. Consistent with previous reports, Der's basal GTPase activity was stimulated by potassium (24, 39). The rate also increased with added KPO_4 and did not increase with additional NaCl, so the stimulation was from the potassium ion as has been reported (data not shown) (24, 39). Surprisingly, we discovered that the stimulatory activity of YihI was inversely proportional to potassium stimulation. Without added KCl, YihI increased the weak GTPase rate of wild-type Der approximately 3-fold. At concentrations of potassium greater than ~250 mM, the stimulation by YihI was lost. Curiously, YihI suppressed the additional stimulation observed at >250 mM potassium (Fig. 5B; see also the supplemental material). Therefore, YihI stimulation is sensitive to potassium concentration. Viewed another way, YihI helps Der function at lower potassium levels.

YihI fails to bind to or restore the GTPase activity of the Der mutants. Using wild-type Der, we sought to establish an affinity between these factors under our standard assay conditions (100 mM KCl) by monitoring the increase in Der's GTPase activity as a

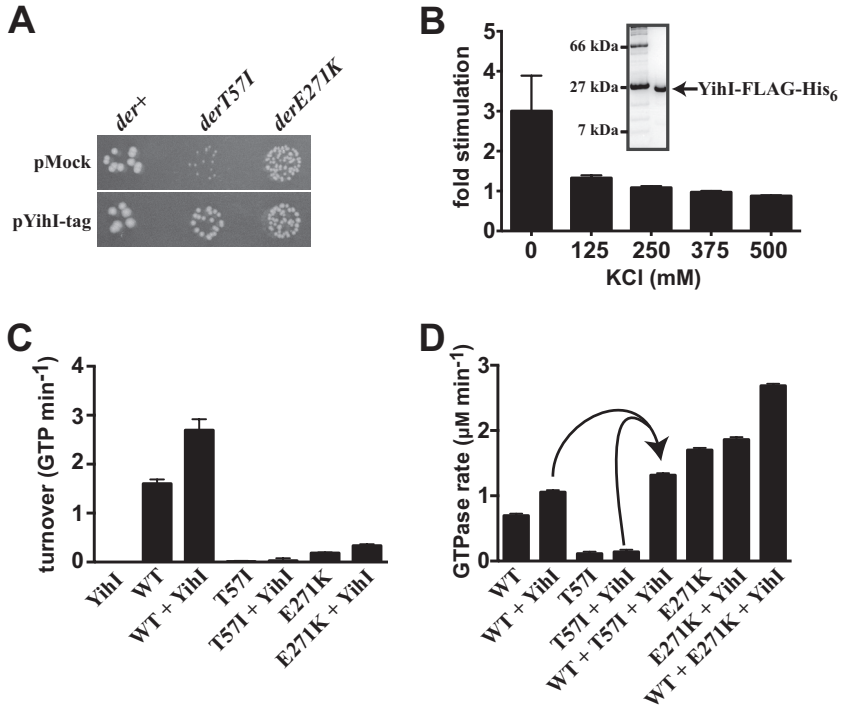

FIG 5 YihI complementation and stimulation of Der. YihI with a FLAG-His_6 tag on its C terminus was expressed from a plasmid and used for complementation studies and to overexpress the protein for purification. (A) L9 strains with wild-type *der*, *derT57I*, or *derE271K* alleles. YihI complementation was evaluated under noninducing conditions to reduce YihI toxicity. YihI expression partially complemented the *derT57I* and *derE271K* mutants but did not restore wild-type growth. (B) The stimulation of wild-type Der (0.5 μM) with and without YihI-FLAG-His_6 (5.0 μM) was measured with increasing KCl and is presented as fold stimulation. At high concentrations of potassium, YihI did not stimulate Der. (C) YihI was added to GTPase assays containing wild-type (0.5 μM) or mutant (2 μM) Der at a 10-fold molar excess in buffer containing 100 mM KCl. No significant stimulation of the T57I mutant and a slight activation of the E271K mutant were observed. (D) Under conditions that allowed approximately half-maximal YihI stimulation of 0.5 μM wild-type Der (YihI at 2.6 μM), GTPase activities were assayed in the presence of either the T57I or E271K mutant as a competitor (each at 5.0 μM). The observed activity of the wild type mixed with the T57I mutant was the sum of the stimulated wild type and the nonstimulated T57I mutant, indicating that T57I did not appreciably compete for YihI (arrows). The E271K mutant was partially stimulated by YihI, and the mixture displayed the sum of both stimulated rates.

function of YihI concentration. Consistent with the previous report of YihI activity under similar conditions (43), we observed a ~50% increase in Der's GTPase when nearly saturated with YihI (Fig. 5C). We were able to derive a K_d between YihI and Der of 2.6 ± 0.6 μM (see the supplemental material). Thus, the affinity between these factors is moderate and consistent with YihI playing a dynamic regulatory role (43).

Next, we evaluated the ability of YihI to stimulate the L9-dependent Der mutants. We did not detect activation of the T57I mutant at our highest tested concentrations (2 μM Der and 20 μM YihI). The E271K mutant could be stimulated, but only at very high protein concentrations (>2 μM for the E271K mutant and 20 μM for YihI [Fig. 5C]). Moreover, although the E271K mutation did not reduce GTP binding, it responded similarly to YihI as mutants that have GTP binding site alterations (S16A and S216A) (23, 43). Thus, YihI appears to aid in the turnover of the enzyme but cannot restore the GTPase activities of the mutants despite the fact it partially complements the *in vivo* phenotypes.

In previous work, it was shown that YihI does not require G domain 1 for binding (43). We were interested in establishing whether YihI could bind to T57I because this protein has a wild-type G domain 2 and KH domain. Therefore, we performed an in-solution competition experiment between the wild type and the T57I mutant for access to YihI. Using our Der-YihI affinity data as a guide (see the supplemental material), we established a condition where the wild-type enzyme was ~50% occupied by YihI (0.5 μM Der and 2.6 μM YihI). Under this condition, small changes in the available YihI would manifest observable changes in the overall GTPase hydrolysis rate. Because the T57I protein is nearly inactive, if this mutant is capable of binding to YihI to any appreciable extent, an excess of the T57I mutant over the wild type in the mixture should reduce the observed stimulation. We did not observe a reduction in the GTPase stimulation of 0.5 μM wild-type protein using a 5 μM concentration of the T57I mutant as a competitor, and the observed rate was the sum of the stimulated wild-type plus the basal T57I mutant rates (Fig. 5D). Therefore, the T57I mutant had no detectable affinity for YihI. For comparison, adding excess E271K mutant to a reaction mixture containing wild-type Der and YihI caused an increase in GTPase activity that was consistent with both enzymes being stimulated simultaneously. Because Der can function with T57 mutated (when second-site suppressed), these results emphasize a new importance of this highly conserved residue aside from routine GTP hydrolysis.

Purified L9 does not influence Der's GTPase activity. When purified L9 was added to assays containing wild-type or mutant Der, there was no change in the GTPase rates. Moreover, L9 did not influence Der that was under YihI stimulation (see the supplemental material). This finding suggests that L9's complementation activity in the *der* mutants may be indirect. Alternatively,

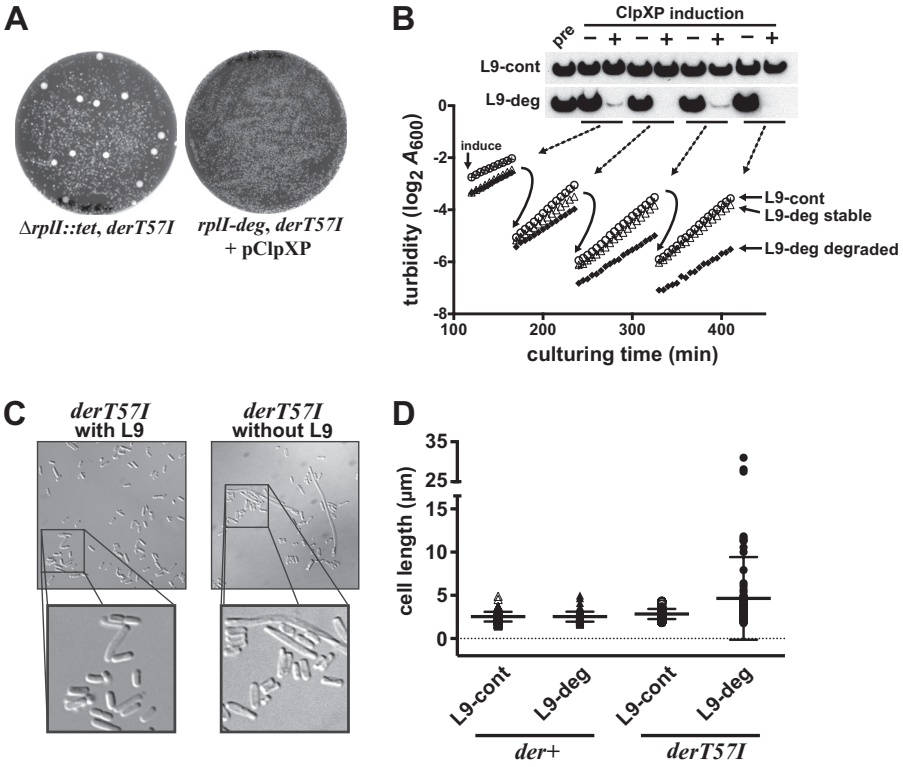

FIG 6 Conditional L9 degradation reveals an unsuppressed *derT57I* phenotype. A Δ*clpX derT57I* strain supported with either L9-cont or L9-deg was transformed with a controllable ClpXP expression plasmid and maintained under noninducing conditions to reduce the accumulation of second-site suppressors. (A) Providing L9-deg to the *derT57I* strain greatly reduced the accumulation of second-site suppressors. On the left is a representative plate showing the presence of suppressed mutant contaminants when the Δ*rplI derT57I* strain was grown as an overnight culture without L9 support. On the right is a plate of *rplI-deg derT57I* Δ*clpX* cells containing pClpXP that were grown from an overnight culture to late exponential phase in glucose medium (ClpXP off) and then plated on arabinose to induce ClpXP and degrade L9-deg. All colonies were small and reminiscent of freshly isolated, unsuppressed Δ*rplI derT57I* strains. (B) Cultures of L9-cont and L9-deg were grown to exponential phase and then either treated with glucose (to repress ClpXP expression [circles and triangles]) or induced with arabinose (to express ClpXP [crosses and diamonds]). As each fast-growing culture neared the end of exponential phase, aliquots of each were diluted 10-fold into fresh medium to allow extended outgrowth. Separate aliquots were removed for Western analysis of the tagged L9 (top). L9-cont was stable and L9-deg was reduced to very low levels by the first sampling. The growth rate of the culture undergoing L9 degradation was reduced by 36% during the last outgrowth. (C) DIC micrographs of *derT57I* strains grown with L9 (L9-cont, pClpXP induced) or without L9 (L9-deg, pClpXP induced). Degradation of L9 caused the cells to become elongated. (D) The lengths of 100 cells from each of four different cultures grown with pClpXP induced for three outgrowths were measured from several micrographs and plotted along with their averages (long lines) and standard deviations. Average lengths (μm): L9-cont, *der*$^+$, 2.53; L9-deg, *der*$^+$, 2.53; L9-cont, *derT57I*, 2.84; L9-deg, *derT57I*, 4.65.

the purified L9 may not be active or appropriately presented to Der (technical limitations prevented us from testing ribosomes with and without L9 at sufficiently high concentrations for these assays).

L9 suppresses an elongated cell morphology caused by *derT57I*. The synthetic-lethality analyses revealed that the small-colony phenotype arises when the L9 support plasmid is lost (Fig. 2A). On the surface, this observation could be interpreted in two ways: either L9 accelerated the growth rate of all *der* mutant cells in a colony (synthetic sickness) or there was a high mortality rate in the *der* mutants and L9 improves survivability (true synthetic lethality). L9 had the greatest influence on the phenotype of cells with the *derT57I* allele, so we focused on this mutant for viability and morphology studies.

We were unable to grow homogeneous cultures of the highly compromised Δ*rplI derT57I* mutant because of the high frequency of second-site suppression (Fig. 6A, left plate). We devised a solution to this problem by employing a targeted protein degradation system to rapidly deplete L9 protein from *derT57I* cells at a convenient time (29). By allowing L9 to suppress the *derT57I* allele during culturing, we were able to grow cultures of sufficient size for biochemical analyses. For this experiment, we modified the *derT57I* strain in three ways. First, we introduced a functional allele of *rplI* that encoded a degradation tag on the C terminus of L9 that is recognized by the processive ClpXP protease (*rplI-deg*); second, we deleted the chromosomal *clpX*; and third, we introduced a controllable ClpXP expression plasmid. A control strain had a tag on L9 that is not recognized by ClpX (*rplI-cont*).

We prepared a dilution of an *rplI-deg derT57I* culture grown with the proteolysis system off and plated it under conditions with the proteolysis system on. All of the colonies were small and reminiscent of freshly isolated *derT57I* strains (Fig. 6A, right plate). Thus, the L9-deg protein was capable of suppressing the slow-growth phenotype sufficiently to allow culturing without the accumulation of fast-growing suppressors.

In an *rplI-deg derT57I* culture, inducing expression of ClpXP caused a rapid depletion of L9-deg (Fig. 6B). Keeping in mind that L9 was still being expressed at high levels as a ribosomal protein, this result indicates that the degradation system was capable of overcoming the L9 synthesis rate and substantially reducing the

half-life of the target protein. We noted that extended induction of ClpXP also caused a slight reduction in the L9-cont levels as well, indicating that the protease exhibited partial activity for this tag. This finding also suggests that L9 protein levels may not be autoregulated.

The thorough depletion of L9 occurred by ~30 min, but we did not observe a pronounced reduction in growth rate until ~4 subsequent mass doublings had occurred (Fig. 6B). This result is important because it demonstrates that ribosomes (or other important factors) synthesized in the presence of L9 and DerT57I are functional when L9 is removed. Thus, DerT57I likely functions in a difficult biogenesis step that, once overcome, no longer requires the support of L9. In addition, this experiment formally establishes that the protein product of *rplI* (and not its mRNA) is responsible for the suppression of this *der* allele.

In a separate set of experiments, we determined that the degradation of L9 in *derT57I* caused a loss in plating efficiency to ~40% that of controls (see the supplemental material). We initially attributed this observation to a loss in cell viability. However, microscopic analyses revealed that culturing of the L9-depleted *derT57I* cells invoked an aberrant, elongated cell morphology (Fig. 6C). The average cell length of L9$^+$ *derT57I* cells was similar to that of L9$^+$ *der*$^+$ cells (2.84 versus 2.53 μm, respectively). In contrast, when L9 was depleted from the *derT57I*, the average cell length increased to 4.65 μm, with a high variance, and the distribution of lengths formed clusters, with some cells being longer than 30 μm (Fig. 6D). Thus, the reduction in plating efficiency was likely caused by a reduction in cell division and not from growth inhibition *per se*. Overall, L9 appears to suppress a cell division defect caused by a crippled Der.

DISCUSSION

We identified mutations in the highly conserved and essential GTPase Der that cause a dependence on ribosomal protein L9 for improved fitness. The T57I mutant was independently recovered and mapped twice during our screen, probably as daughters of the original mutant that separated during recovery. This mutant displayed a pronounced phenotype and illustrates a balance between the inactivation of an essential enzyme and the ability to identify potential candidates during the visual screening of the library. In G domain 1, T57 is located within the G3 motif, which connects to the switch II region of this GTPase. Threonine is found at this position in nearly all Der orthologs and could play an important role in the function of the invariant flanking motif residues.

G domain 1 is reported to be responsible for the majority of Der's GTPase activity (23, 39), so breaking the basic catalytic mechanism may explain the highly defective nature of this mutant. However, this GTPase domain is thought to undergo a dramatic reorganization during the GTP hydrolysis cycle. Structures of the Der ortholog from *Bacillus subtilis* show the G domain rotated such that the T57 location is positioned far away from the KH domain (39). In contrast, in the *Thermotoga maritima* structure, T57 sits at a well-packed interface between G domain 1 and the RNA-binding KH domain (37). Therefore, the T57I mutation may interfere with the ability of the domain to properly interact with the KH domain. The numerous second-site suppressors of T57I also support an architectural role for this residue, because if it is required for GTP hydrolysis, only revertants should have functioned well.

The E271K mutation sits in the switch II motif of G domain 2 (38). Although there is generally a high variability in switch domains of GTPases (44–46), E271 appears invariant among all Der proteins. Switch motifs in GTPases are thought to couple the energy of GTP hydrolysis to the movement of the switches and allow the enzymes to do mechanical work (44). In the crystal structures of Der, E271 is not in direct contact with residues of the P-loop that gates the GTP hydrolysis site. Nonetheless, this residue is situated at a location that docks this switch against Der in the GTP-bound state. Envisioning a tensioned spring that gets released by GTP hydrolysis during the cycling of the enzyme, a mutation at this location could prevent the formation of a stable, high-energy state of the switch. Our observation that the affinity for GTP of the E271K mutant was comparable to that of the wild type also supports the idea that GTP binding was not inhibited but that the cycling of the enzyme through high- and low-energy states was compromised. Perhaps even more compelling is the observation that the GTPase activity of G domain 1, which is reported to possess the majority of the observed GTPase activity (37), was also substantially inhibited by this mutation.

We observed partial complementation of the phenotypes of each mutant by overexpressing YihI. This factor was identified as an interacting partner of Der that stimulated Der's GTPase rate by both lowering the K_m and increasing the V_{max} (43). Because YihI was not observed to stimulate GDP release, it was designated a GAP-like factor (43). Unlike canonical GAPs, YihI is reported to stimulate Der only marginally, and our stimulation data were consistent with this conclusion. One interpretation of these findings is that Der is not likely to be a signaling GTPase, so raising its basal GTPase rate several orders of magnitude, as traditional GAPs do, would not be warranted (47). Alternatively, YihI may not perform a bona fide GAP function by contributing to catalysis and could stimulate the GTPase activity by stabilizing a catalytically active conformation, an idea put forth by its discoverers (43).

We did not observe *der* mutant complementation by YihI that was better than that provided by L9, suggesting that the growth defects caused by the *der* mutant persisted in the presence of excess YihI. Moreover, YihI is not universally conserved in bacteria, and it is nonessential (43), yet overexpression is toxic (suggesting that it can shield Der from important targets). Der has joined a growing list of GTPases that require high potassium levels for optimal activity (24, 39). We discovered that YihI had no stimulatory activity when potassium was present at high levels. Interestingly, the potassium level in *E. coli* and *Klebsiella pneumoniae* (in which YihI is present) is reported to fluctuate between ~100 and ~250 mM, whereas those of *B. subtilis* (in which YihI is absent) are maintained at ~400 mM (39, 48–50). Perhaps YihI helps load Der with potassium or helps Der function when intracellular potassium levels are low.

The goal of this project was to decipher why L9 is conserved in nature, so what is L9's role in Der physiology? Part of the conundrum stems from the fact that we do not know what Der specifically does. Der depletion for extended periods causes the accumulation of unstable and/or incomplete large subunits and defects in both 16S and 23S rRNA processing, which others have suggested points to a role in ribosome biogenesis (23–26, 51). Cells with deficient rRNA folding factors commonly display cold sensitivities (46, 52). We tested for cold sensitivity in our *der* mutants and observed none (data not shown). In addition, another group reported that overexpression of the stringent response factor RelA suppressed the growth defects caused by Der with mutations in

either GTPase site, but not a *der* null mutation (53). A conclusion from that project was that the overexpressed RelA increased (p)ppGpp pools, restricted rRNA synthesis, and restored balance to the assembly process. We tested for the ability of overexpressed RelA to suppress the L9-dependent Der mutants, and despite imparting a growth restriction in all strains, we observed no relative fitness increases in the mutants (data not shown). Thus, the T57I and E271K mutants are distinct from Der variants with defective GTPase centers in this regard.

An interesting feature of the large ribosomal subunits recovered from Der-depleted *E. coli* is that they are sensitive to reduced magnesium levels, suggesting that they have not been assembled correctly (25, 26). Curiously, when these destabilized subunits were evaluated for protein content, L9 was among the few proteins reported to fall off (25, 26). This finding suggests that the binding site of L9 may be compromised when Der activity is reduced. In line with this notion, our studies indicate that the N-terminal domain alone is able to complement *derT57I* as well as the full-length protein. Additionally, the *hop-1* mutation in L9 is in a conserved patch on the C domain, and this variant complements our Der mutants as well as the wild type (data not shown). Considering that there are extensive contacts between the N domain and the 23S RNA, it seems that this portion of L9 may help stabilize the large subunit when Der activity is limiting.

The physiological functions of Der and L9 remain a mystery. An additional approach we took to interrogate the role of L9 in the Der mutants was to evaluate translation bypassing in the mutants using established reporters. Aside from the complication of second-site suppression, we discovered a curious phenomenon that led us to abandon that approach (see the supplemental material).

We used a targeted degradation system to get around a thorny genetic problem and to preliminarily interrogate the physiology of Der mutants as they lose the support of L9. We were pleased to find that the degradation system could deplete L9 so well considering that it is a highly expressed protein that is tightly associated with the ribosome. We plan to use our degradation system to evaluate the integrity of ribosomes built with a defective Der upon L9 depletion both *in vivo* and *in vitro*. In the preliminary investigation reported here, we revealed an elongation phenotype when the *derT57I* strain lost the support of L9. We also observed elongated cells in unsupported cultures of *derE271K* organisms (data not shown). We interpret these results as a problem with cell division caused by a defective Der and not necessarily a problem with biomass accumulation. Thus, *der* mutants were likely recovered in our screen because the loss of L9 promotes the retention of the reporter plasmid by reducing cell division. These findings raise interesting new questions about the roles of L9 and Der in ribosome assembly and in maintaining bacterial physiologies.

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