The interaction of an essential *E. coli* GTPase, Der with 50S ribosome via the KH-like domain

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Running title: The role of C-terminal KH-like domain of Der

Key words: Der, GTPase, KH domain, ribosome, *Escherichia coli*

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Abstract

An essential *E. coli* tandem GTPase Der has been implicated in 50S subunit biogenesis. The *rrmJ* gene encodes a methyltransferase that modifies U2552 residue of 23S rRNA, and its deletion causes a severe growth defect. Peculiarly, overexpression of Der suppresses growth impairment. In this study, using an *rrmJ*-deletion strain, we demonstrate that two GTPase domains of Der regulate its association with 50S subunit via the KH-like domain. We also identified critical region of Der for its specific interaction with 50S subunit.
Emerging evidences are that many *E. coli* GTPases play critical roles in ribosome biogenesis (6). For example, *E. coli* Era consists of a conventional GTP-binding domain and a KH domain (an RNA-binding domain) with a consensus VIGXXGXXI sequence (9). The direct interaction between Era and 16S rRNA was demonstrated by structural studies of *Thermus thermophilus* 30S ribosomal subunit complexed with Era (24). Peculiarly, Era was shown to suppress the cold-sensitive cell growth of the *rhlA*-deletion strain of which gene product resembles a KH domain and plays an important role in 30S subunit assembly at low temperature (13, 16). A unique GTPase subfamily of Der (double Era-like GTPase, also known as EngA) is conserved only in eubacteria. We have previously demonstrated that Der is cofractionated with 50S subunits in a GTP-dependent manner and that Der plays a critical role in 50S subunit maturation at a later biogenesis step (15).

Interestingly, both GTP-binding domains (G-domains) were essential for cell growth, and moreover, two G-domains function cooperatively, suggesting that GTP-induced conformational changes and GTPase activity are essential for cell viability as well as function (1, 15). The X-ray crystal structures of two Der orthologs from *Thermotoga maritima* and *B. subtilis* revealed that the C-terminal domain shares a similar topology with a KH domain without a consensus sequence motif and is flanked by two G-domains (22, 23). It was suggested that the GTP-bound form of YphC (a Der ortholog in *B. subtilis*) triggers a dramatic conformational change, which favors an interaction with negatively charged ribonucleic acids by exposing a positively charged KH-like domain with a high pI value (14, 22).
The overexpression of *E. coli* Der functionally suppressed the slow growth defect of a deletion strain of the *rrmJ* gene, whose gene product is a methyltransferase modifying at the U2552 residue in the A-loop of 23S rRNA in an intact 50S subunit (5, 26). Even though ∆*rrmJ* (strain HB23) is viable, it causes a serious defect on cell growth by accumulating 50S and 30S ribosomal subunits at the expense of 70S ribosomes. Thus, overexpression of Der seems to overcome its weak interaction with 50S subunits unmethylated at U2552. In this study, using an ∆*rrmJ* strain as a genetic background, we tried to elucidate the functional regulation between two G-domains and the KH-like domain. We further characterized the KH-like domain by random mutagenesis, and identified crucial residues for its association with 50S subunit. Our data suggest that the unique C-terminal domain indeed plays a role in rRNA/ribosome recognition.

**Both G-domains of Der are associated with its suppression of ∆rrmJ.**

Previously, it was shown that DerN118D, DerN321D, DerS16A and DerS216A could not support the cell growth of *der*-deletion strain (1, 15). However, it is unknown if the suppression of ∆*rrmJ* (strain HB23) is associated with G-domains of Der. Therefore, we introduced seven *der* alleles including wild-type and six different G-domain mutants respectively into ∆*rrmJ* to test their suppression effect. First, the open reading frames of *der* and *rrmJ* were digested with NdeI and EcoRI and cloned into the NdeI-EcoRI site of pIN^A^ (an IPTG-inducible plasmid, Amp^r^) (10), yielding pIN^A^EcDer and pIN^A^RrmJ, respectively. The mutant plasmids were constructed by site-directed mutagenesis, yielding pIN^A^EcDerGD-1, pIN^A^EcDerGD-2, pIN^A^EcDerGD-12, pIN^A^EcDerGA-1, pIN^A^EcDerGA-2 and pIN^A^EcDerGA-12. These clones express DerN118D, DerN321D, DerN118D/N321D, DerS16A, DerS216A and DerS16A/S216A, respectively. Both Asn
and Ser residues are conserved in the motif sequence of the conventional G-domain (14), and their mutations to Asp or Ala effectively inhibit GTPase activity (1, 15, 23, 27). The ΔrrmJ strain was transformed with plasmids described above, and the transformed cells were plated on LB ± IPTG plates containing ampicillin followed by incubation at 30°C. On both plates, plasmid pIN^EcDer was able to fully suppress the null phenotype of ΔrrmJ as pIN^RrmJ expressing wild-type RrmJ (Fig. 1A). Plasmids pIN^EcDerGD-1 and pIN^EcDerGD-2, however, partially suppressed ΔrrmJ by forming smaller colonies. However, cells transformed with pIN^EcDerGD-12 did not show any suppression effect at all. Unlike Asn to Asp mutations, none of pIN^EcDerGA-1, pIN^EcDerGA-2 and pIN^EcDerGA-12 plasmids suppressed the growth defect of ΔrrmJ under the condition, suggesting that the Ser to Ala mutation in G-domains is more inhibitory than the Asn to Asp mutation. Furthermore, we tested whether truncated forms of Der are able to restore the null phenotype of ΔrrmJ. pIN^EcDerΔN and pIN^EcDerΔC were constructed by truncating the first G-domain or the entire C-terminal domain of Der, respectively. Plasmid pIN^EcDerΔN or pIN^EcDerΔC was transformed into ΔrrmJ and transformants were incubated at 30°C. Both transformants were not able to support the cell growth, suggesting that both the N-terminal G-domains and the C-terminal domain are indispensable (Fig. 1A). For the comparisons of suppression effects caused by Der variants, cell growths were measured in a liquid medium. In this experiment, IPTG was not added for the following reasons; i) Hager et al. observed that in case of RrmJ, the leaky expression was enough to restore cell growth (12). We observed that the leaky expression of not only RrmJ but also Der is able to restore growth of ΔrrmJ, and ii) we wanted to observe the different effects caused by moderate leaky expression of various
mutant proteins (Fig. 1B). It clearly demonstrates that plasmids pIN\textsuperscript{A}EcDerGD-1 and pIN\textsuperscript{A}EcDerGD-2 partially suppress the phenotype of \textit{ΔrrmJ} but not pIN\textsuperscript{A}EcDerGD-12.

**Inability of G-domain mutant Der to interact with 50S ribosomal subunits.**

Next, we examined the GTP analog-dependent 50S subunit association of Der in those partially suppressed cells. Cells transformed with wild-type or mutant plasmids were grown in LB medium at 30°C and cell lysates were prepared in the presence of a GTP analog, GDPNP. First, polysomes from each strain were separated by 5-40% sucrose density gradient sedimentation. Cells harboring a control vector accumulated a substantial amount of ribosomal subunits with concomitant reductions of polysomes and 70S ribosomes (approximately 12% 70S, 55% 50S and 33% 30S, Table 1), while the suppressor strain containing pIN\textsuperscript{A}EcDer recovered a normal ribosome profile (36% 70S, 42% 50S and 22% 30S) as demonstrated by Tan et al. (2002). The value for 70S ribosomes increased to 37.2% with pIN\textsuperscript{A}RrmJ in the absence of IPTG. When RrmJ was induced by the addition of IPTG, the value further increased to 51.6% (Table 1). Notably, partially suppressed cells harboring either pIN\textsuperscript{A}EcDerGD-1 or pIN\textsuperscript{A}EcDerGD-2 accumulated less ribosome subunits than pIN\textsuperscript{A} transformants (Table 1). The cells expressing the double mutant DerN118D/N321D showed the same polysome defect as the \textit{ΔrrmJ} cells harboring pIN\textsuperscript{A} (Fig. 1C).

Subsequently, using fractions of each sucrose gradient, Western blot analysis of Der proteins in \textit{ΔrrmJ} was carried out to detect the association of Der proteins with 50S subunit. In two strains harboring vector only or pIN\textsuperscript{A}RrmJ, endogenous Der was co-localized with 50S subunit. Note that more Der proteins are associated with 50S subunits in cells harboring pIN\textsuperscript{A}EcDer than pIN\textsuperscript{A}RrmJ. It also revealed that the associations of
DerN118D, DerN321D and DerN118D/N321D to 50S subunit were significantly diminished compared to that of wild-type Der (Fig. 1D), even though a large amount of 50S subunits was accumulated in the polysome profile (Fig. 1C). It seems that only endogenous Der was detected at 50S subunit fractions of ∆rrmJ harboring plN^ΔEcDerGD-1, plN^ΔEcDerGD-2 or plN^ΔEcDerGD-12, suggesting a very weak or limited binding of G-domain mutant Der proteins to 50S subunit. These data indicate that ∆rrmJ suppression by Der requires intact two G-domains.

Isolation of KH mutants Der by random mutagenesis. As described briefly above, the binding of GTP to Der may trigger substantial conformational changes, exposing KH-like domain to solution, which in turn promotes its ribosome interaction (22). Thus, in order to explore that the C-terminal domain of Der is responsible for ribosome interaction, we used the ∆rrmJ genetic backgrounds to screen KH-domain mutants. We created random mutations by PCR specifically on the C-terminal region of der by incorporating dITP. The mutated PCR fragments and pIN^ΔEcDer were digested with ClaI-EcoRI, and the doubly digested PCR fragments were ligated into the ClaI-EcoRI site of pIN^ΔEcDer. Then, the resulting ligation mixture was transformed into ∆rrmJ. Transformants with a slower growth rate at 30°C were screened as mutant Der, and pIN^Δ and plN^ΔEcDer were used as a negative and positive control, respectively, to compare the growth rate. Three candidates were initially screened as defective mutants and all three clones expressed the full-length Der proteins confirmed by Western blot (data not shown). Each plasmid contained a combination of three mutations, the first plasmid contained R468L, N469K and K488R, the second contained Q396H, V400A and G414R, and the third contained F352L, G424D and T472A, respectively. To dissect
which mutation is responsible for slow cell growth, a site-directed mutagenesis for each
of the nine mutations was carried out and nine mutant plasmids were transformed and
tested for growth rate in ΔrrmJ. ΔrrmJ harboring pIN^A^EcDer or pIN^A^RrmJ normally
formed colonies on LB ± IPTG plates, and among nine mutations, F352L, Q396H, V400A, R468L and K488R did not affect the colony formation. However, cell growth of
ΔrrmJ was not supported by pIN^A^EcDerG414R, pIN^A^EcDerG424D, pIN^A^EcDerN469K
or pIN^A^EcDerT472A (Fig. 2A). Cell growth in a liquid medium was further monitored to
quantitate the growth rate of each transformant. Consistently, ΔrrmJ cells harboring
pIN^A^EcDerG414R, pIN^A^EcDerG424D, pIN^A^EcDerN469K or pIN^A^EcDerT472A grew
slowly, especially, pIN^A^EcDerG424D showed the same growth phenotype as ΔrrmJ (Fig.
2B). These data indicate that those four residues Gly414, Gly424, Asn469 and Thr472
may play important roles in the function of the C-terminal domain.

**KH mutant Der proteins are impaired in polysome recovery.** In order to
examine if these four residues are directly involved in the association of KH-like domain
with 50S subunit, we prepared cell lysate samples in the presence of GDPNP and
analyzed the polysome and ribosomal subunit profiles. Sucrose gradient centrifugation
was carried out at 10 mM Mg^{2+} concentration as in Fig. 1C. All the ΔrrmJ cells
expressing KH mutant Der proteins accumulated both 50S and 30S subunits with a
reduced amount of 70S and polysomes (Fig. 3A and Table 1). The ΔrrmJ expressing
wild-type RrmJ or Der recovered the normal polysome profiles as described in Fig. 1C.
DerG424D mutant showed the most severe polysome impairment compared to
DerG414R, DerN469K and DerT472A proteins. Subsequently, sample fractions of
sucrose gradient were subjected to Western blot analysis as shown in Fig. 3B. It revealed
that the mutants, DerG414R, DerG424D, DerN469K and DerT472A substantially diminished its association with highly accumulated 50S subunits, suggesting that each mutation interrupted the associations of Der to 50S subunits, which, in turn, caused the accumulations of ribosomal subunits in ∆rrmJ. Note that since all four mutant proteins have two intact G-domains; it is the KH-like domain that plays a major role in the ribosome binding of Der.

Accumulation of aberrant 50S ribosomal subunits in ∆rrmJ. Der-depleted cells accumulated aberrant 50S subunits in vitro at different Mg²⁺ concentrations as described previously (15), and the ∆rrmJ cells also accumulated ~40S ribosomal subunits at a low Mg²⁺ concentration (3). Next, we tested if ∆rrmJ accumulates aberrant 50S subunits at various Mg²⁺ concentrations. The ∆rrmJ cells were grown in LB medium and cell pellets were resuspended in a buffer containing 0.25, 0.5 or 1 mM of Mg²⁺. Cell lysates were then loaded to 5-25% sucrose gradient containing 0.25, 0.5 or 1 mM Mg²⁺, respectively followed by ultracentrifugation. 50S subunits of ∆rrmJ migrated at four different positions depending on Mg²⁺ concentrations (Fig. 3C). At 1 mM Mg²⁺, normal 50S and abnormal 50S (line a) appeared, and at the lower Mg²⁺ concentrations (0.5 and 0.25 mM), two aberrant 50S subunits (lines b and c, respectively) were detected with a substantially reduced amount of normal 50S subunits. Note that 30S subunits remain unaffected under the conditions used. We subsequently analyzed ribosomal subunit profiles of ∆rrmJ expressing DerG414R, DerG424D, DerN469K and DerT472A. Expression of RrmJ or wild-type Der suppress the abnormality of 50S subunit, while those four KH mutant Der proteins could not suppress the accumulation of aberrant 50S subunits as in Fig. 3D. These results suggest that DerG414R, DerG424D, DerN469K and
DerT472A proteins cannot stabilize 50S subunits of ∆rrmJ cells at low Mg^{2+}
concentration. Therefore, it is likely that both Der and RrmJ may contribute to the
integrity of 50S subunits likely through a common mechanism.

Since the overexpressed DerG414R, DerG424D, DerN469K and DerT472A
proteins significantly reduced the binding activity to unmethylated U2552 of 23S rRNA,
next, we tested if the methylation at U2552 affects the association with mutant Der
proteins. For this purpose, first, mutant alleles, der(G414R), der(G424D), der(N469K)
and der(T472A), were ligated into pIN vector (same as pIN^A except a chloramphenicol-
resistant cassette) yielding pINEcDerG414R, pINEcDerG424D, pINEcDerN469K and
pINEcDerT472A. Additionally, since Asn469 and Thr472 residues are located within the
last 30 amino acid residues of E. coli Der, which is not found in T. maritima Der
(TmDer), pINEcDer460 encoding Der (1-460 aa; Der460) was cloned in order to
examine the effect of the truncation on ribosome association. These mutant plasmids
were transformed into the der-deletion strain in which U2552 of 23S rRNA is methylated.
This strain harbors a helper plasmid carrying the der gene, a temperature-sensitive
replication origin and an ampicillin-resistant gene (15). Transformants were plated on LB
plates containing ampicillin, chloramphenicol and kanamycin, and plates were incubated
at 30°C. Colonies were picked and streaked on LB plates containing chloramphenicol and
kanamycin, and then plates were incubated at 42°C to remove a helper plasmid. All of
transformants were not able to form colonies at 42°C on LB plates containing ampicillin,
confirming the loss of a helper plasmid (data not shown). As shown in Fig. 4A,
pINEcDerG414R, pINEcDerN469K and pINEcDerT472A complemented the der-
deletion strain at 42°C; however, colony formation of the der-deletion strain was
negatively affected by pINEcDerG424D and pINEcDer460 plasmid. These results imply that methylation at U2552 residue of 23S rRNA in intact 50S subunits induces a significant local rearrangement around methylation site.

Peculiarly, Der460 was unable to support cell growth of a der-deletion strain; therefore, in order to further analyze the inability of Der460 to complement the der-deletion strain, pINEcDer or pINEcDer460 was transformed into wild-type E. coli cells and we performed polysome analysis followed by Western blot experiments. Surprisingly, in contrast to full-sized Der (Fig. 4B, panel a), Der460 interacts with polysomes in the presence of GDPNP as well as ribosomal subunits (Fig. 4B, panel c). Note that the endogenous Der (arrow head) co-exists in 50S subunit fractions with Der460. Together, it is possible that both Der proteins might be present at different 50S subunits or the binding site of Der460 to 50S partially overlaps with that of full-length Der in the presence of GDPNP. Next, we tested if this nonspecific interaction of Der460 with polysomes is nucleotide dependent. We carried out the same experiments as shown in Fig. 4B, panels a and c except that GDPNP was replaced with GDP or no nucleotide (Apo state). To a lesser extent, Der460 still associated with polysomes. These data imply that the KH-like domain including the last 30 amino acid residues determines its specific association with 50S subunit.

As shown in Fig. 1, both two G-domains and the KH-like domain are essential for viability in ΔrrmJ as well as the der-deletion strain (1, 15) and those G-domain mutant Der proteins have an impaired ribosome association. All G-domain mutant Der proteins, Der GD-1, Der GD-1 and Der GD-12 substantially reduced a binding activity to 50S
subunit (Fig. 1D), which supports the idea that cooperative nucleotide bindings to Der
stimulate ribosome association.

As mentioned earlier, upon nucleotide binding, Der appears to undergo
conformational rearrangements for its ribosome association (22, 23). The KH mutant Der
proteins isolated in this study have both G-domains intact and notably; those KH
mutations did not alter the GTPase activity of Der (data not shown). From the
comparisons of those KH mutations with TmDer and YphC structures, the mutations are
located in the positively charged β-sheets (β14 and β15, respectively) of the KH-like
domain (22, 23). Thus, Gly414 and Gly424 residues may be directly involved in Der
association with ribosome in the ΔrrmJ cells. Alternatively, mutations at those residues
may cause conformational changes in Der to inhibit its interaction with ribosomes. On the
basis of TmDer X-ray structure (23), GD1 of E. coli Der is assumed to interact with the
C-terminal 30 amino acid residues of Der that protrude toward GD1 in the structure. Thus,
it is tempting to speculate that upon binding of GTP, the KH-domain becomes released to
specifically interact with 50S subunits.

Methylation at U2552 takes place at the A-loop of 23S rRNA that makes a direct
interaction with aminoacyl (A)-site tRNA, and the function of RrmJ, thus, was implicated
in translation efficiency as well as accuracy (4, 28). Unlike other 23S rRNA
mehtyltransferases such as RmA, RlmB, RumA and RumB that are dispensable (11, 19,
20), RrmJ seems to be crucial for ribosome function, probably because of the temporal
and spatial importance of methylation at U2552 (3). Methylation at U2552 substantially
modifies the A-loop fold of 23S rRNA (2), and interestingly, KH mutant Der proteins are
not defective in the der-deletion strain, suggesting that overexpressed Der may recognize
the structural changes of the A-loop region unmethylated at U2552 in ∆rrmJ (Figs. 2 and 4).

50S subunits accumulated in ∆rrmJ were extremely unstable at lower Mg²⁺ concentration with underrepresented L5, L16, L18, L25, L27, L28, and L30 (12). Der-depleted cells also accumulated aberrant 50S subunits with a low amount of L2, L6, L9 and L18, suggesting that both Der and RrmJ are critical for ribosome assembly and integrity, and share a mechanism to stabilize 50S subunits at a very late stage of 50S subunit maturation (15). These ribosomal instabilities were dependent on Mg²⁺ concentration in vitro (Fig. 3C and 15). Ribosomes, especially, rRNAs, extensively interact with Mg²⁺ ions that neutralize the negative charges of rRNA backbone (17), and starvation of Mg²⁺ ions has a negative impact on the integrity and assembly of ribosome (7, 8, 21). This may explain why starvation of Mg²⁺ in the absence of either RrmJ or Der in E. coli causes disintegration of 50S subunits.

Concluding remarks. In this study, we demonstrated that GTP-induced conformational change of Der is very important for its function on 50S subunit, and by using the ∆rrmJ strain we elucidated that the KH-like domain is required for ribosome recognition. At present, it is unknown how Der and RrmJ proteins contribute to the structural integrity of 50S subunits in cells. Further studies of Der may provide us an insight into the role of Der in ribosome biogenesis. Due to the unique primary sequences and topology of the KH-like domain in Der, it may be possible to design antibiotics that inhibit the binding of the KH-like domain to 50S subunit.

We thank Drs. Sangita Phadtare and Ursula Jacob for their scientific insights and kind gift of strain ∆rrmJ (HB23).
REFERENCES


Figure legends

FIG. 1. Phenotypes of ∆rrmJ expressing G-domain and mutant Der. (A) The ∆rrmJ (strain HB23) cells transformed with plasmids were grown at 30°C overnight in LB medium containing ampicillin (50 µg/ml), and cultures were diluted. The diluted cultures were streaked on LB ± 1 mM IPTG (Isopropyl-β-D-thiogalactopyranoside) plates containing ampicillin. GD-1, GD-2, GA-1 and GA-2 indicate N118D, N321D, S16A and S216A mutations, respectively. GD-12 and GA-12 indicate the double mutations. (B) Growth curves of ∆rrmJ harboring different plasmids. Cells were cultured in LB medium containing ampicillin at 30°C and cell cultures were diluted five times before OD\textsubscr{600} measurement. (C) Polysome profiles of wild-type and G-domain mutant Der in ∆rrmJ.
The ∆rrmJ cells harboring various plasmids were cultured to log phase at 30°C in LB medium containing ampicillin. Polysomes were trapped by the addition of chloramphenicol to the culture to a final concentration of 0.1 mg/ml. The cell pellets were resuspended with a buffer BP [20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NH₄Cl, and 5 mM β-mercaptoethanol]. GDPNP was added at a final concentration of 100 µM. Approximately 10 A₂₆₀ units of each cleared extract was layered onto a sucrose gradient. The preparation of cell lysates and polysomes analysis was carried out by ultracentrifugation in a Beckman SW41 rotor for 3.5 h at 210,000 × g, as described previously (15). (D) Association of wild-type and G-domain mutant Der with 50S subunit in ∆rrmJ. 16 µl of individual gradient fractions from (C) was subjected to 12.5% SDS-PAGE analysis followed by Western blot using anti-Der antisera (15).

FIG. 2. (A) Screening of KH mutant Der proteins and their phenotypes in ∆rrmJ. The C-terminal part of the der gene was randomly mutagenized by PCR as described in Lerner et al. (18) and Spee et al. (25). Each of the four dNTPs (dATP, dCTP, dGTP, and dTTP) was depleted one at a time in PCR. MnCl₂ (0.5 mM) and dITP (200 µM) were also included in the reaction, and error-prone Taq polymerase was used. Plasmids with mutation were transformed into ∆rrmJ and transformants were streaked on LB ± 1 mM IPTG plates containing ampicillin. (B) Growth curves of ∆rrmJ transformed with KH mutant plasmids. Cell culture and optical density measurement were carried out as in Fig. 1B.

FIG. 3. Ribosome profiles of ∆rrmJ expressing KH mutant Der. (A) The ∆rrmJ cells harboring the indicated plasmids were cultured at 30°C in LB medium containing ampicillin, and polysomes were resolved by ultracentrifugation as carried out in Fig. 1.
(B) Cofractionation of mutant Der with 50S subunits in ∆rrmJ. SDS-PAGE analysis and Western blot were carried out as in Fig. 1D. Western blots of three controls were taken from Fig. 1D. (C) Ribosomal subunit profiles of ∆rrmJ. The cell pellets were resuspended with a buffer BP containing 0.25, 0.5 or 1 mM MgCl₂. Polysomes were resolved by ultracentrifugation for 3.5 h at 230,000 × g. Three lines (a, b and c) indicate aberrant 50S subunits with three different migrations. Arrows indicate 50S subunits. (D) Ribosomal subunit patterns of ∆rrmJ expressing RrmJ, Der and KH mutant Der. The cell pellets were prepared as described in (A), and resuspended in buffer BP containing 0.25 mM MgCl₂. Subunits were resolved by ultracentrifugation. An arrow head indicates the position of aberrant 50S subunits.

FIG. 4. Phenotypes of der-deletion strain expressing KH mutant Der. (A) The der-deletion strain was transformed with pINEcDer, pINEcDerV400A, pINEcDerG414R, pINEcDerG424D, pINEcDerN469K, pINEcDerT472A or pINEcDer460, and plated on LB plates containing chloramphenicol (40 µg/ml), and kanamycin (35 µg/ml). Plates were first incubated at 30°C, and colonies formed at 30°C were streaked on LB plates containing chloramphenicol and kanamycin, and plates were then incubated at 42°C. (B) The non-specific interaction of Der460 with ribosome. The wild-type cells harboring pIN²EcDer or pIN²EcDer460 were cultured and the polysome samples were prepared in the presence of GDPNP, GDP or Apo state. Polysome analysis and Western blot were carried out as described in Fig. 1. The wild-type cells harboring pIN²EcDer or pIN²EcDer460 were used as controls in the first two lanes of Western blot. An arrow head indicates the endogenous Der in 50S subunit fractions, and the numbers indicate the end of each fraction.
Table 1. The amounts of free 30S, 50S and 70S ribosomes in ∆rrmJ cells expressing RrmJ or Der variants.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Ribosome(^a) (%)</th>
<th>70S</th>
<th>50S</th>
<th>30S</th>
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<tr>
<td>pIN(^A)</td>
<td>12.3</td>
<td>55.2</td>
<td>32.5</td>
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<tr>
<td>pIN(^A)RrmJ</td>
<td>37.2</td>
<td>39.6</td>
<td>23.3</td>
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<tr>
<td>pIN(^A)RrmJ+ 1mM IPTG</td>
<td>51.6</td>
<td>23.9</td>
<td>24.4</td>
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<tr>
<td>pIN(^A)EcDer</td>
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<td>41.6</td>
<td>22.2</td>
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<td>pIN(^A)EcDerGD-1</td>
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<td>45.5</td>
<td>29.6</td>
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<tr>
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<td>54.8</td>
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<td>NA</td>
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<td>NA</td>
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<td>48.7</td>
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<td>pIN(^A)EcDerT472A</td>
<td>26.8</td>
<td>48.2</td>
<td>25.1</td>
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</table>

\(^a\) Values represent the average amount of free 30S, 50S and 70S ribosomes from duplicate experiments.

\(^b\) and \(^c\) The value of 70S was not available.
Figure 2

A

B

-Time (h)
0 2 4 6 8 10
OD600
0.0
0.2
0.4
0.6
Vec.
RrmJ
Der
Der G414R
Der G424D
Der N469K
Der T472A
Der K488R
Der F352L
Der Q396H
Der T472A -IPTG +IPTG

-Time (h)
0 2 4 6 8 10
OD600
0.0
0.2
0.4
0.6
Vec.
RrmJ
Der
Der G414R
Der G424D
Der N469K
Der T472A
Der K488R
Der F352L
Der Q396H
Der T472A
Figure 3

A

Der G414R

50S

70S

30S

polysomes

Der N469K

Der G424D

Der T472A

B

Vec.

RrmJ

Der

Der G414R

Der N424D

Der N469K

Der T472A

C

1 mM Mg^{2+}

0.5 mM Mg^{2+}

0.25 mM Mg^{2+}

ab c

50S

30S

D

Vec.

RrmJ

Der

Der G414R

Der G424D

Der N469K

Der T472A
Figure 4

A

B

a, Der+GDPNP

polysomes 70S 50S 30S

d, Der460+GDP

b, Der+GDP

c, Der460+GDPNP

d, Der460+GDP

e, Der460+Apo