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# Investigating the In Vivo Activity of the DeaD Protein Using Protein–Protein Interactions and the Translational Activity of Structured Chloramphenicol Acetyltransferase mRNAs

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**Abstract** Here, we report the use of an in vivo protein–protein interaction detection approach together with focused follow-up experiments to study the function of the DeaD protein in *Escherichia coli*. In this method, functions are assigned to proteins based on the interactions they make with others in the living cell. The assigned functions are further confirmed using follow-up experiments. The DeaD protein has been characterized in vitro as a putative prokaryotic factor required for the formation of translation initiation complexes on structured mRNAs. Although the RNA helicase activity of DeaD has been demonstrated in vitro, its in vivo activity remains controversial. Here, using a method called sequential peptide affinity (SPA) tagging, we show that DeaD interacts with certain ribosomal proteins as well as a series of other nucleic acid binding proteins. Focused follow-up experiments provide evidence for the mRNA helicase activity of the DeaD protein complex during translation initiation. DeaD overexpression compensates for the reduction of the translation activity caused by a structure placed at the initiation region of a chloramphenicol acetyltransferase gene (*cat*) used as a reporter. Deletion of the *deaD* gene, encoding DeaD, abolishes the translation activity of the mRNA with an inhibitory structure at its initiation region. Increasing the growth temperature disrupts RNA secondary structures and bypasses the DeaD requirement. These observations suggest that DeaD is involved in destabilizing mRNA structures during translation initiation. This study also provides further confirmation that large-scale protein–protein interaction data can be suitable to study protein functions in *E. coli*. J. Cell. Biochem. 100: 642–652, 2007. 

**Key words:** SPA-tagging; protein interaction network; helicases; DEAD-box proteins; translation initiation

Assigning putative functions to proteins based on their interactions with each other is a well-established method in studying functional proteomics [Pandey and Mann, 2000; von Mering et al., 2002; Bader et al., 2003]. Protein–protein interactions can be most effectively identified using pull-down experiments, yeast two-hybrid analysis, or purifying protein complexes that have been tagged in vivo. Tagging protein complexes in vivo and then purifying

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them by affinity chromatography has the advantage of identifying those complexes that really exist in vivo (provided that the tagged protein is not overproduced). To study protein–protein interactions in *Escherichia coli*, we have recently applied homologous recombination of a linear PCR product into the chromosome to attach two affinity tags, 3X FLAG and a calmodulin-binding peptide, separated by a tobacco etch virus (TEV) protease cleavage site, to the C-terminus of a target gene product [Zeghouf et al., 2004]. This method is called sequential peptide affinity (SPA) tagging and results in native levels of tagged protein in the cell. The affinity purification procedure is non-denaturing leading to the purification of native protein complexes. Also, there is no overproduction of the tagged protein. This significantly reduces the chance of false positives. The subunits of the purified protein complexes as well as any other interacting proteins are identified by mass spectrometry (MS).

Very recently we showed that these affinity-tagging methods can be performed in *E. coli* cells on a genome-wide scale [Butland et al., 2005]. Here, we report that SPA-tagging can be further used to study the function of DeaD (stands for DEAD-box containing) protein in *E. coli*.

The DeaD protein, encoded by the *deaD* gene, belongs to a family of RNA helicases that are characterized by the presence of a DEAD motif [Linder et al., 1989]. Certain members of the DEAD family of proteins are known to be involved in translation, pre-mRNA splicing, ribosome assembly, and other essential processes. The DeaD protein is shown to be required for the in vitro reconstitution of protein synthesis complex formation from pure components indicating the importance of this protein for translation of certain mRNAs [Ganoza et al., 2002].

The DeaD protein was originally isolated as a multi-copy suppressor of an *E. coli* mutant for *rpsB* genes, which encodes the S2 ribosomal protein [Toone et al., 1991]. It was later illustrated that DeaD has an in vitro RNA helicase activity [Jones et al., 1996]. Recently it was shown that the in vitro helicase activity of DeaD is ATP dependent [Bizebard et al., 2004].

The function of the DeaD protein during translation initiation has been studied in vitro with several types of mRNA templates [Lu et al., 1999]. The authors suggested that only the translation of the structured mRNAs, and not those lacking structures, were dependent on the DeaD protein. The formation of mRNA-70S-fMet-tRNA complex on structured mRNAs either occurred extremely inefficiently or not at all in the absence of the DeaD protein. Addition of the DeaD protein restored the formation of mRNA-70S-fMet-tRNA on these mRNAs [Lu et al., 1999].

The eukaryotic (eIF-4A) counterpart of the DeaD protein has also been isolated and its in vitro activity on translation has been investigated in detail. It was shown that the protein unwinds the secondary structure of the mRNA initiation leader regions, three to five nucleotides at a time, enabling formation of a complex between the mRNA and the initiator-tRNA [Hershey and Merrick, 2000]. The protein requires the presence of an RNA-binding factor, eIF-4B, to processively unwind the RNA double-stranded regions [Hershey and Merrick, 2000].

In spite of some understanding of the reactions in vitro, little is known about the action of the DeaD protein in the cell. A series of experiments have indicated that the overexpressed *deaD* gene stabilizes the mRNA transcripts [Iost et al., 1994]. In these experiments, synthesis of β-galactosidase was measured as a reporter gene to a number of mRNAs. When the *E. coli* host RNA polymerase was replaced by the faster bacteriophage T7 enzyme for the transcription of the *lac*Z gene, the yield of the transcripts was found to be reduced by about 100-fold. Overexpression of the DeaD protein greatly increased the low yields of the *lac*Z mRNAs by stabilizing the transcripts [Iost et al., 1994]. In another series of experiments, it was shown that the overexpression of DeaD protein restores binding of certain ribosomal proteins to the ribosomes [Moll et al., 2002]. The authors suggested that the DeaD protein might play a role in ribosome biogenesis [Moll et al., 2002]. In a recent report, deletion of the *deaD* gene resulted in a deficit in free 50S ribosomal subunits at low temperatures along with the accumulation of a new 40S particle [Charollais et al., 2004]. It was concluded that the DeaD protein is involved in 50S ribosomal subunit biogenesis [Charollais et al., 2004]. It has also been shown that the disruption of the *deaD* gene (also called *csdA* for cold-shock DEAD-box protein A) resulted in altered expression of

certain genes at low temperatures [Jones et al., 1996]. The expression of the DeaD protein was induced at low temperatures, at which, the DeaD protein was the major ribosome-associated protein [Jones et al., 1996]. Ribosomes are proposed to be the physiological sensors for the cold-shock response and certain translation factors, IF-2α and IF-2β, are known to be cold-shock proteins [Jones et al., 1996].

Here, we expand upon our previous protein–protein interaction detection approach by purifying tagged DeaD in the presence of ribonuclease (RNase) in order to distinguish those protein–protein interactions, which are mediated by RNA. We further use focused genetic and biochemical experiments to study the function(s) of the *deaD* gene.

## MATERIALS AND METHODS

### Protein Tagging

The details of the one-step PCR transformation tagging in *E. coli* are explained elsewhere [Zeghouf et al., 2004]. Tagged cells were grown at 32°C and the tagged complexes were affinity purified as before [Butland et al., 2005] with the following exception. When required, the interference of the DNA and RNA molecules were eliminated by the addition of 2 μg/ml and 5 U/ml final concentrations of RNase A and benzonase, respectively, to the sonication buffer before affinity purification, unless stated otherwise. Each protein complex purification experiment was repeated at least three times. Only those proteins identified to interact with the DeaD protein in multiple purifications are recorded in Table I.

**TABLE I. List of the Proteins Found to be Interacting With the DeaD Protein in Multiple Purifications**

| Interacting protein | Property |
|---|---|
| RpsA<br>RpsB<br>RpsC<br>RpsD<br>RpsE<br>RpsF<br>RpsG<br>RpsM<br>RpsO<br>RpsT | Proteins associated with 30S ribosomal subunits, bind 16S rRNA |
| RplA<br>RplB<br>RplC<br>RplD<br>RplM<br>RplV | Proteins associated with 50S ribosomal subunits, bind 23S rRNA |
| YfiF | Hypothetical tRNA and rRNA methylase with a SpoU RNA methylase/binding domain |
| YgiF[a] | Hypothetical protein with adenylate cyclase domain |
| YcbY | Oxidoreductase with methylase activity on adenines associated with DNA |
| SrmB | A DEAD-box RNA helicase, contains ATP and RNA-binding domains |
| VacB | Ribonuclease R with RNase II and S1 RNA-binding domains |
| YhiR | Hypothetical protein with DNA methylation domain |

The majority of these proteins possess nucleic acid binding activities, emphasizing that the DeaD protein couples and works in conjunction with proteins that bind to nucleic acids. The DeaD interacting proteins can be further grouped into 30S and 50S ribosomal subunit associated proteins, and others. The observed interactions were validated by reciprocal SPA-tagging of YfiF in which the DeaD protein was identified as an interacting protein. After the degradation of RNA and DNA molecules in the cell extract, only the interaction between the DeaD protein and YgiF was altered indicating that the majority of the observed DeaD complex interactions are mediated by protein–protein interactions.
[a]Addition of RNase A and benzonase to the cell extract eliminated this interaction.

### Bacterial Strains and Expression Plasmids

The bacterial strain in which the *deaD* gene (*deaD*Δ) has been deleted [Charollais et al., 2004] was a generous gift from Dr. Isabelle Iost (CNRS, France). Plasmid NT5-deaD used to overexpress the DeaD protein in the *E. coli* cells was constructed as follows. Plasmid pUC-deaD carrying *deaD* gene (a generous gift from Dr. M. Dreyfus, CNRS, France) was digested by *Bsp*HI and *Hind*III and blunted by Klenow. The released fragment was inserted into the unique *Sma*I site of the expression plasmid pGEX-4T-1. In this way the expression of the recombinant DeaD fused to a glutathione S-transferase (GST) tag is under the transcriptional control of an inducible *tac* promoter. The orientation of the insert was determined using restriction enzyme digestion and the content of the first 100 nucleotides of the insert was verified by DNA sequencing.

To make chloramphenicol acetyltransferase (cat) mRNA constructs, synthetic double-stranded oligonucleotides were purchased from Gibco BRL. The primary sequences of the fragments are shown in Figure 1A. The oligonucleotides contain an *Xho*I and a *Hind*III overhangs at each end. The overhangs were used to force clone the fragments in a pBR322-derived expression plasmid [Golshani et al., 2000b], in front of a *cat* gene and under the transcriptional control of a constitutive promoter P1 (Fig. 1B). The translation

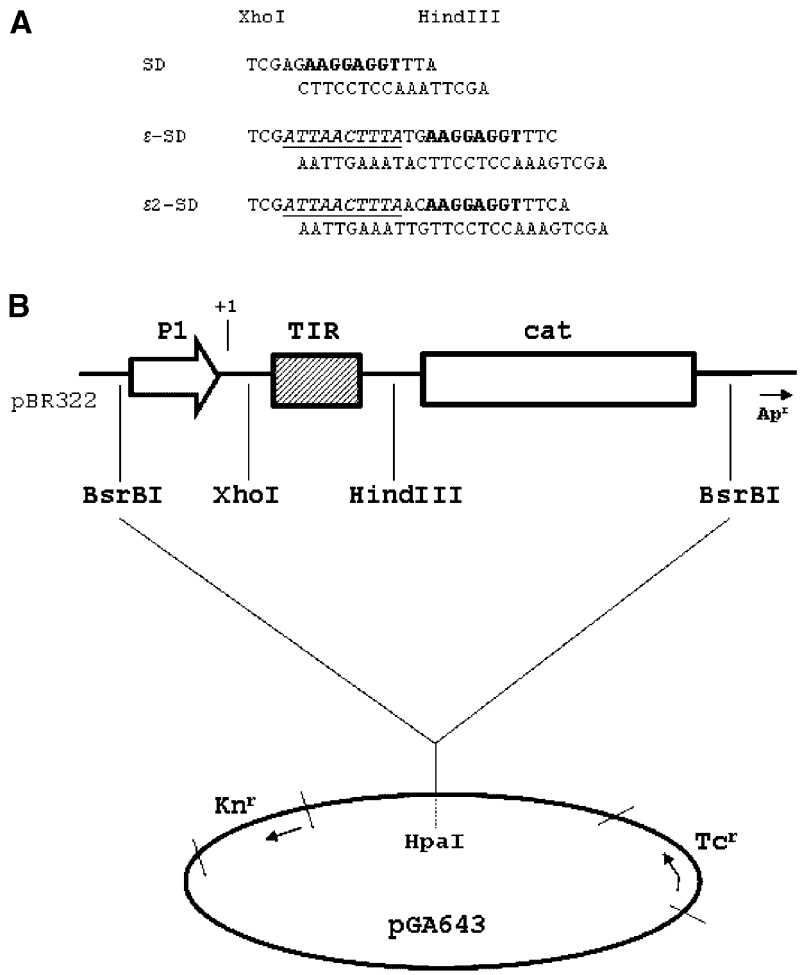


**Fig. 1.** Nucleotide sequences (**A**) and expression plasmids (**B**) used for producing *cat* mRNAs with various structures at their translation initiation regions (TIR). A: Synthetic oligonucleotides containing epsilon (in italics and underlined letters) and Shine-Dalgarno (SD) (in bold letters) sequences. B: Structures of the pBR322 and pGA643 derived expression plasmids. The expression cassette consists of a constitutive promoter (P1), cloning site for TIR, and a reporter (*cat*) gene. The *Xho*I and *Hind*III overhangs of the oligonucleotides were used to force clone the fragments in a pBR322-derived expression plasmid in front of the *cat* gene. The expression cassettes (P1, TIR, and *cat* gene) were released from the pBR322 plasmid using *Bsr*BI digestion and cloned into the *Hpa*I site of a pGA643-derived plasmid.

initiation region (TIR) of the cat mRNA was removed. The construct thus made has an ampicillin (Ap) resistant character and a conditional (depending on the translation initiation activity of the cloned fragment) chloramphenicol (Cm) resistance. The content of P1 promoter, the cloned oligonucleotides, and the first 100 nucleotides of the *cat* gene were verified using DNA sequencing. The series of expression plasmids derived in this manner were used to study the effect of the deletion of the gene encoding DeaD on the activity of cat mRNAs. To study the effect of the overproduction of DeaD on the expression of cat mRNAs, the complete expression cassette containing the P1 promoter, cloned fragments, and the *cat* gene were released from the pBR322 plasmid using *Bsr*BI digestion and cloned into the *Hpa*I site of a pGA643-derived plasmid [Golshani et al., 2000b] as shown in Figure 1B. The plasmids made in this way have a tetracycline (Tc) and a kanamycin (Kn) resistant character and a conditional (depending on the translation initiation activity of the cloned fragment) Cm resistance. The orientation and content of the cloned fragment was verified using restriction digestion and DNA sequencing.

### cat Assay

To study the effect of the DeaD overexpression on cat mRNA translation, *E. coli* BL21 cells co-transformed with pGA643-derived cat

expression constructs together with either NT5-deaD (which overexpresses the DeaD protein) or NT5 (as control), were cultivated in LB (Luria-Bertani) medium supplemented with 50 μg/ml Ap and 8 μg/ml Tc at 25°C until early stationary phase was reached. Two $OD_{595}$ units of cells were spun down, washed with the wash buffer (10 mM Tris-HCl pH 7.0, 150 mM NaCl), resuspended in 1 ml of TE buffer (10 mM Tris-HCl pH 7.0, 1 mM EDTA, 0.1% SDS), and disrupted using a French Press. The cell lysate was centrifuged and the cat activity was measured spectrophotometrically [Shaw, 1975; Golshani et al., 2000a] and related to the total bacterial protein measured by a Bradford-Kit (Biorad). The cat activity was estimated by averaging the activities obtained from five independent experiments.

To study the effect of the absence of the DeaD protein on the cat mRNA translation, *deaD*Δ cells were transformed with pBR322-derived cat expression constructs and cultivated at 25°C (unless otherwise indicated). The cat activity measurements were performed as above.

### Protein and mRNA Analysis

For Western blot analysis, 50 μg of total *E. coli* protein (prepared as above) was subjected to electrophoresis on a 12% polyacrylamide–SDS gel. Proteins were transferred onto nitrocellulose filters and incubated with rabbit antisera raised against purified yeast eIF-4A. The filters were then treated with a secondary (anti-rabbit) IgG conjugated with alkaline phosphatase. The relative content and half-life of cat mRNA was determined by dot blot using a $^{33}$P-labeled primer (GCCCATGGTGAAAACGGGGGG) specific to cat mRNA in three independent experiments as described elsewhere [Ivanov et al., 1992]. The relative activity of each RNA spot was determined by a phosphor-imager (Biorad).

## RESULTS

### In Vivo Tagging of *deaD* Gene, Affinity Purification, and Identification of the Associated Proteins

The *deaD* gene was tagged in *E. coli* and affinity purified as previously reported [Butland et al., 2005]. The expression of the tagged gene was verified by a Western blot using antibodies against the tagging module (data not shown). The complexes associated with the tagged DeaD protein were purified and their protein components were identified by MS. Figure 2A,B shows a silver-stained SDS–PAGE of the affinity-purified DeaD complex. Polypeptides visible on the gel after purification from the tagged strain, which were absent in the parallel control preparations from an untagged strain (Fig. 2E), were identified using MALDI-TOF MS. In this way the relative stoichiometry of co-purifying protein may be estimated. A second portion of the purified protein complexes was subjected to trypsin digestion and directly analyzed by LC-MS/MS in order to identify small or less abundant proteins. The list of the proteins identified to interact with the DeaD protein in multiple purifications is shown in Table I. Treatment of the protein complexes with RNase A (2 μg/ml) and benzonase (5 U/ml) prior to the affinity purification step was used to distinguish those interactions, which are independent of the RNA and DNA intermediate molecules (Fig. 2B). This treatment eliminated the interaction between DeaD and YgiF only, indicating that the majority of the observed interactions appear independent of the nucleic acid molecules.

The nuclease treatment condition used here is not exhaustive. Therefore, it remains possible that after this treatment, some small nucleic acid molecules as well as regions of the ribosomes that are more resistant to nuclease treatment may remain intact and hence, mediate some of the observed interactions. To address this, more vigorous nuclease treatment conditions using 10 U/ml benzonase in addition to 5 μg/ml and 10 μg/ml RNase A (Fig. 2C,D, respectively) were used. It was observed, however, that the presence of higher concentration of nucleases in the purification buffer interfered with the affinity capture of the SPA-tagged DeaD protein. This suggests a limitation for the SPA-tagging in which all traces of the nucleic acid molecules may not be completely eliminated from the purified complex.

The majority of proteins which were found to interact with the DeaD protein also possessed a nucleic acid binding activity, emphasizing that the DeaD protein couples and works in conjunction with proteins that bind to nucleic acids (Table I). This is in agreement with the observation that the eukaryotic (eIF-4A) counter part of the DeaD protein interacts and works in conjunction with the RNA-binding protein eIF-4B. To confirm the observed inter-

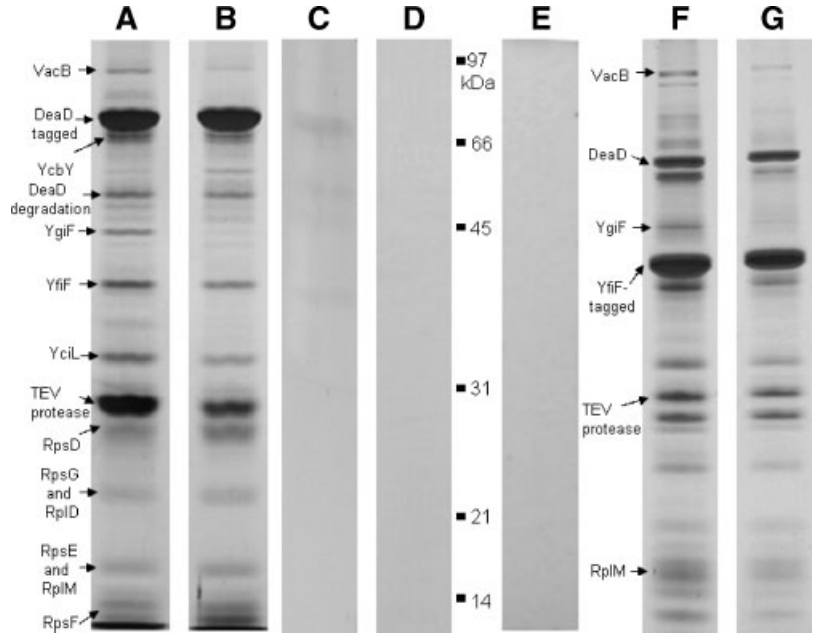


**Fig. 2.** Isolation of protein complexes containing the DeaD protein. The protein complexes are affinity-purified and analyzed by an SDS–PAGE followed by silver staining. Individual subunits of the purified complexes, recognized by distinct bands on the gel, were identified by in-gel trypsin digestion followed by MALDI-TOF MS. The protein purification was carried out with *E. coli* strains containing the SPA-tagged DeaD (**A**) and YfiF (**F**) proteins or no tag (**E**). In Figure 2, (**B**) and (**G**) are similar to (A) and (F), respectively, with the exception that in (B) and (G), RNase A (2 μg/ml) and benzonase (5 U/ml) were added to the cell extraction (sonication) buffer. In Figure 2, (**C**) and (**D**) are similar to (A) with the exception that they contain 10 U/ml benzonase in addition to 5 μg/ml and 10 μg/ml of RNase A, respectively. In (A), the band corresponding to YgiF was absent in (B) suggesting that the observed interaction with YgiF is mediated via an RNA/DNA intermediate. The remaining interactions seemed to be similar between the two purifications (A and B). The presence of higher concentrations of nucleases (C and D) interfered with the purification of the SPA-tagged DeaD. The observed interactions in (A) and (B) were validated by reciprocal SPA-tagging and purification of YfiF (F and G). In the YfiF purifications (F and G), the DeaD protein was identified as an interacting partner.

actions, reciprocal protein purification was performed using SPA-tagged YfiF protein, which was originally identified in association with the DeaD protein. As expected the DeaD protein was observed to interact with the SPA-tagged YfiF protein confirming the validity of the observed interactions (see Fig. 2). As an internal control an untagged *E. coli* strain was subjected to SPA purification (Fig. 2E). No protein was isolated in the absence of a SPA tag, indicating that the purification is tag specific.

The proteins, which interacted with DeaD, can be further grouped into two categories: the ribosomal-associated proteins and those independent of the ribosomes (Table I). Ribosomal subunits can be found in free forms, the 30S (harboring 21 proteins) and the 50S subunits (harboring 33 proteins), or in association with one another to form the 70S ribosome. During translation, the 30S subunit binds to mRNA and fMet-tRNA forming a pre-initiation complex. The 50S subunit then joins the 30S-mRNA-fMet-tRNA complex to form an initiation complex. The initiation complex then enters a round of translation. The fact that the relative number of proteins in DeaD complexes, which are associated with 30S ribosomal subunits, is significantly higher than those associated with the 50S subunit suggests that the activity of DeaD protein is more closely coupled to the activity of the 30S subunit. This in turn indicates that the DeaD protein may play a role during mRNA and fMet-tRNA binding to the ribosomes, and hence, during translation initiation. These data are in agreement with our previous observation that DeaD protein is present in the 30S and 70S ribosomes, but not in the 50S subunits (Aoki and Ganoza, unpublished data).

Besides the observed interactions with the ribosome-associated proteins, the DeaD protein also interacted with certain non-ribosomal nucleic acid binding proteins. These proteins are thought to bind nucleic acids and play various roles with respect to nucleic acid

modifications. For example, YfiF is a hypothetical RNA-binding protein with an RNA methyl transferase activity, VacB is thought to bind RNA and has an RNase activity, YcbY is an oxidoreductase which binds DNA and methylates adenine residues, etc. The fact that DeaD interacts with proteins with various nucleic acid binding and modifying activities suggests that the DeaD protein may function to complement the activity of the associated proteins. A nucleic acid helicase activity for DeaD fits very well with this observation. A helicase can destabilize various structures so that the modifying enzymes may perform their methylation, degradation, binding, and other activities. This is in agreement with an interaction between the eukaryotic (eIF-4A) counter part of DeaD and the RNA-binding protein eIF-4B [Hershey and Merrick, 2000].

The relative stoichiometry of co-purifying proteins also fits well with a helicase activity for DeaD. The low abundance of the interacting proteins may indicate transient interactions, which is expected for a helicase involved in many independent tasks.

All together, the observed interactions may suggest a general helicase activity for DeaD protein. This general helicase activity also seems to have a role during translation initiation.

### The Helicase Activity of DeaD in *E. coli* Cells

To investigate the helicase activity of DeaD in vivo, three constructs were made (Fig. 1A and 4). The Shine-Dalgarno (SD) construct contains a SD sequence as a translation initiator 5′ to the AUG start codon of a cat reporter mRNA. The ε-SD construct carries a translation enhancer sequence known as epsilon or ε, 5′ to the SD sequence. In the ε2-SD construct, the ε sequence of ε-SD is positioned such that a strong inhibitory secondary structure may occur at the initiation region (see Fig. 3). All these constructs are under the transcriptional control of a strong constitutive (P1) promoter (see Fig. 1). cat activity measurement was used to determine the efficiency of each of the constructs to initiate translation under the physiological levels of the DeaD protein as well as under conditions in which DeaD was overexpressed (Table II).

The results presented in Table II compare the translational activity of the cat transcripts at a physiological level of DeaD to that when DeaD is overexpressed. Since in certain cases the overexpression of DeaD is known to stabilize the half-life of certain mRNAs, the cat activity measurements were normalized to the content of the mRNAs. As expected it was observed that when ε sequence was not involved in a secondary structure (ε-SD), it enhanced

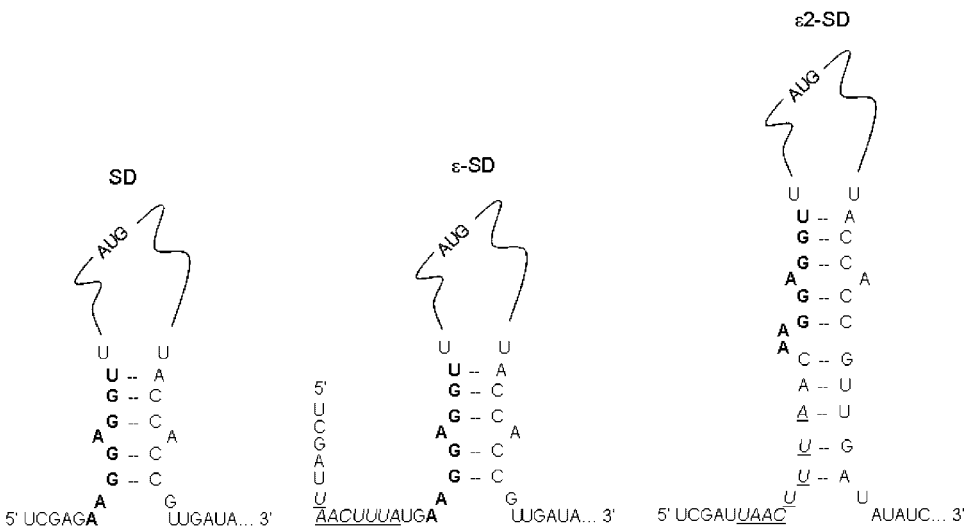


**Fig. 3.** The potential secondary structure of the TIRs of *cat* mRNA constructs. The SD sequence is in bold letters and the epsilon sequence is in italic and underlined letters. The structures were predicted by mfold and their free energies were estimated from RNA pairing free-energy decomposition tables for different temperatures. The estimated values are −5.2 kcal/mol and −6.8 kcal/mol for ε-SD construct at 37°C and 25°C, respectively, and −8.6 kcal/mol and −11.5 kcal/mol for ε2-SD at 37°C and 25°C, respectively.

**TABLE II. Effect of the Overexpression of DeaD on cat mRNA Translation**

| mRNA (pGA643 derived) | Wild-type (containing plasmid NT5) | | | DeaD overexpressed (containing plasmid NT5-deaD) | | |
|---|---|---|---|---|---|---|
| | Relative cat activity[a] | Relative cat mRNA content[b] | Normalized cat yield[c] | Relative cat activity | Relative cat mRNA content | Normalized cat yield |
| SD | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| ε-SD | 2.88 | 1.42 | 2.03 | 3.11 | 1.51 | 2.06 |
| ε2-SD | 1.28 | 1.12 | 1.14 | 2.53 | 1.29 | 1.96 |

The ε sequence (ε-SD) enhances the translation mediated by a Shine-Dalgarno sequence (SD). When the ε sequence is hindered by an inhibitory structure (ε2-SD), its enhancing activity is highly reduced. Overexpression of the DeaD protein restores the enhancing activity of the ε sequence indicating a possible role for the DeaD protein in destabilizing the inhibitory structure. The stability (half-life) of the cat mRNAs ranged from 65 to 75 (±5) s.
[a]The cat activity is related to the activity of the SD construct, which is measured to be 1,031 ± 196 U/mg and 970 ± 112 U/mg of bacterial protein for the wild-type and DeaD overexpressed cells, respectively. The data represent the average from five independent experiments.
[b]The mRNA content is related to that of the SD construct and represents the average values from three independent experiments with an error range of 20% or less.
[c]The data for cat yield are normalized to the cat mRNA content by dividing the relative cat activity by the relative cat mRNA content.

translation. The enhancing activity of ε became highly reduced when ε was involved in a secondary structure (ε2-SD; see Fig. 3). When DeaD was overexpressed, however, the efficiency of the ε sequence involved in a secondary structure (ε2-SD) was restored. It was therefore concluded that the overexpression of DeaD may destabilize the secondary structure at the initiation region of ε2-SD and thus make this sequence more accessible for translation.

To further show that the DeaD protein complex is responsible for destabilizing secondary structures at the TIRs, the translation initiation activities of the three constructs: SD, ε-SD, and ε2-SD were compared in a wild-type *E. coli* strain and in the strain in which the gene for DeaD was deleted (*deaD*Δ). The results shown in Table III indicate that in the *deaD*Δ strain both the SD and ε-SD constructs were capable of initiating translation with the same efficiency as in the wild-type strain at 25°C. The translation initiation activity of ε2-SD, which contains a stable secondary structure, however, was greatly reduced (more than 10-fold) in the absence of the DeaD protein. These data further support a helicase activity for the DeaD protein during translation initiation.

If DeaD destabilizes the inhibitory secondary structure in the ε2-SD construct then it may be possible to compensate for its absence by an increase in the growth temperature. Therefore, the translation initiation activities for the above constructs were re-examined at a higher temperature. The cat activity measurements

**TABLE III. Effect of the Deletion of *deaD* Gene on cat mRNA Translation**

| mRNA (pBR322 derived) | Wild-type cells | | | *deaD*Δ cells | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 25°C | | | 25°C | | | 37°C | | |
| | Relative cat activity[a] | Relative cat mRNA content[b] | Normalized cat yield[c] | Relative cat activity | Relative cat mRNA content | Normalized cat yield | Relative cat activity | Relative cat mRNA content | Normalized cat yield |
| SD | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| ε-SD | 2.74 | 1.33 | 2.06 | 2.77 | 1.31 | 2.11 | 2.93 | 1.35 | 2.17 |
| ε2-SD | 1.23 | 1.08 | 1.14 | 0.06 | 0.75 | 0.08 | 0.82 | 0.95 | 0.86 |

The cat mRNA containing an SD sequence (SD) is efficiently translated. Addition of the ε sequence to the mRNA (ε-SD) increases its translational activity. The presence of an inhibitory structure on this mRNA (ε2-SD) reduces its translational activity. The absence of the DeaD protein (*deaD*Δ cells) abolishes the translational activity of the mRNA with inhibitory structure only, without altering the translational activities of those mRNAs, which lack inhibitory structures. This suggests that the DeaD protein is required for an efficient translation of the mRNA with an inhibitory structure. The stability (half-life) of the cat mRNAs ranged from 65 to 75 (±5) s.
[a]The cat activity is related to the activity of the SD construct, which is measured to be 2,342 ± 441 U/mg of bacterial protein for wild-type cells, 2,245 ± 512 U/mg and 2,639 ± 488 U/mg of bacterial protein for *deaD*Δ cells grown at 25°C and 37°C, respectively. The data represent the average from five independent experiments.
[b]The mRNA content is related to that of the SD construct and represents the average values from three independent experiments with an error range of 20% or less.
[c]The data for cat yield are normalized to the cat mRNA content as in Table II.

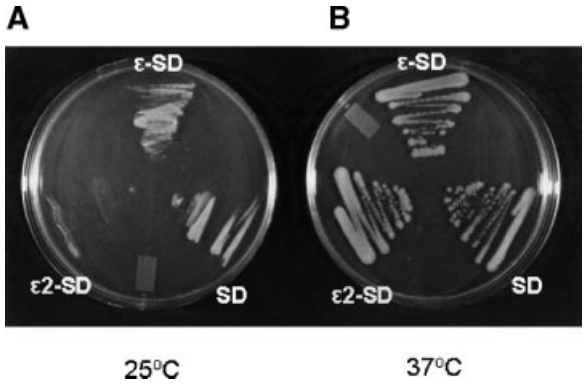


**Fig. 4.** Growthof *deaD*Δ *E. coli* cells in the presence of 100 μg/ml of chloramphenicol (Cm). The strains are transformed with SD (used as a control), ε-SD, or ε2-SD constructs. The cells are grown in either 25°C (**A**) or 37°C (**B**). In the absence of an inhibitory structure on the mRNA (SD and ε-SD) the cells are capable of growing in both temperatures indicating a sufficient level of cat mRNA translation. When an inhibitory structure is placed on the cat mRNA (ε2-SD), cell growth is highly altered at 25°C, indicating that the level of cat mRNA translation is significantly reduced in these cells. When the temperature is increased to 37°C, the cells re-gain their growth. This is presumably due to the destabilization of the inhibitory structure on the cat mRNA by the higher temperature.

shown in Table III indicate that the ε2-SD construct re-gained its translation initiation activity in the *deaD*Δ strain when the growth temperature for the cells was increased from 25°C to 37°C. In agreement with this result the *deaD*Δ strain transformed with one of the constructs SD, ε-SD, or ε2-SD was capable of growing in Cm concentration of 100 μg/ml when incubated at 37°C (Fig. 4). However, when the same *E. coli* cells were placed at 25°C, only those cells transformed with the SD or the ε-SD constructs were capable of growth and not those cells transformed with the ε2-SD (which contains inhibitory structure) construct.

## DISCUSSION

It is generally accepted that proteins accomplish their tasks in association with protein partners, forming stable or transient protein complexes [Pandey and Mann, 2000]. As a consequence, one possible approach to investigate the function of a protein is to examine the proteins with which it interacts. Consequently the identification of protein–protein interaction networks in eukaryotes has received considerable attention over the past few years. One of the approaches to analyze protein–protein interactions is based on the analysis of the protein complexes, which are captured using affinity methods. A well-established example of affinity-captured complexes comes from the proteomics studies in *Saccharomyces cerevisiae*. In this organism, a target protein is first double-tagged on its native chromosomal locus and then captured under physiological conditions. The components of the purified complexes are then identified and the function of the target protein is investigated using the function and the properties of the proteins with which it interacts [Krogan et al., 2003; Wood et al., 2003]. We recently applied this methodology to *E. coli* on a genome-wide scale using a SPA tag [Butland et al., 2005].

Here, we report the extension of this technique to attempt to differentiate between proteins bound in a complex by either protein–protein or protein–RNA interactions. We use this approach to study the in vivo function of the DeaD protein in *E. coli*. The DeaD protein is shown to have an RNA helicase activity in vitro, but its activity in vivo has remained controversial.

Identification of the components of DeaD complex reveals that the DeaD protein interacts with various DNA- and RNA-binding proteins. To confirm these interactions, a reciprocal SPA purification was done using SPA-tagged YfiF strain. DeaD was identified as an interacting partner confirming the validity of the observed interactions. SPA purification of an untagged strain was used as an internal control and indicated that the purification was tag specific. The functions of the proteins which interacted with DeaD are extremely diverse. Some are involved in translation (for example, ribosome-associated proteins), some are modifying enzymes (for example, YcbY), and some are RNases (for example, VacB). RNase treatment of the complex before affinity purification revealed that most of these interactions seem to be independent of mediating RNA/DNA molecules. It remains possible, however, that certain regions of the ribosomes that are more resistant to RNase treatment may mediate some of the observed interactions. The functional diversity of DeaD interacting proteins identified in this manner suggests that DeaD plays more of a general role, which complements the individual activity of each of these proteins. A general nucleic acid helicase protein fits very well with this observation. A helicase can bind the ribosomes and help it destabilize the mRNA inhibitory structures, thus promoting translation from structured templates. A

general helicase can also destabilize inhibitory structures, which prevent modification or degradation of nucleic acid molecules.

Focused follow-up experiments using cat mRNAs with inhibitory structures provided further support for the in vivo helicase activity of the DeaD protein. We find that an increase in the stability of the secondary structure that encompasses the SD region (ε2-SD) significantly reduces the synthesis of the cat protein in *E. coli* cells. Overexpression of the DeaD protein, however, compensated for the observed reduction of cat synthesis from this construct. Consistent with this observation is the fact that the ε2-SD construct re-gained its activity in *deaD*Δ cells when the temperature was shifted from 25°C to 37°C. These results imply that the *deaD* gene product has a role in destabilizing inhibitory structures during the initiation of cat mRNA translation in vivo. From these data, however, it is not clear whether DeaD directly induces structural changes in the TIR. It remains possible that the DeaD protein may recruit other proteins that cause the structural changes to this region. Further studies are required to elucidate the details of such mRNA structural changes. These studies can also include the use of mRNAs other than cat, to confirm the observed helicase activity of DeaD during cat mRNA translation.

Initiation of mRNA translation in prokaryotes requires the presence of a start codon as well as a nucleotide sequence (the SD sequence) that is complementary to the 3′-terminus of the 16S rRNA of the 30S subunit [McCarthy and Brimacombe, 1994; Kozak, 1999; Gualerzi et al., 2000]. These signals are essential for the translation of most transcripts but do not always ensure a proper initiation as they occur with some frequency within the coding regions. It is hypothesized that the authenticity of a translation initiation site can be determined by the relative absence of secondary structures in the initiation region [Hall et al., 1982; Ganoza and Louis, 1993; Ganoza et al., 2002]. Authentic start codons are thought to be located in structure free regions making them more accessible to the translation machinery. It is therefore possible that the DeaD protein functions to ensure a structure free region at the initiation site of an mRNA. Overexpression of the DeaD protein could in principle destabilize mRNA structures and thus promote translation initiation.

The in vivo activity of DeaD protein presented in this study is consistent with our in vitro observations on the mechanisms of DeaD. We have previously shown that the pure DeaD protein was essential for initiation complex formation with mRNAs that harbor secondary structures and was not required for synthesis with mRNAs lacking this feature [Lu et al., 1999]. However, in these experiments as well as in the in vivo experiments presented here, we cannot exclude the possibility that the DeaD protein may also act in structures that occur in the early coding region. Such secondary structures appear to affect initiation of most mRNAs, including mRNAs that lack a leader region. Nevertheless, it is clear that the DeaD protein acts on structures that occur at the initiation region. Since the DeaD protein is conserved throughout species, study of the features of the mRNA that depend on the action of the DeaD protein should result in a better understanding of the mRNA structures required to initiate translation.

This study also provides further confirmation that the large-scale protein–protein interaction data can be suitable to study protein functions in prokaryotes. Consequently, it can set the path for further genome-wide protein mapping projects in other prokaryotic organisms.

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