JOURNAL OF BACTERIOLOGY, June 2009, p. 3706–3711 Vol. 191, No. 11
0021-9193/09/$08.00+0 doi:10.1128/JB.01434-08

The Chaperone GroESL Enhances the Accumulation of Soluble, Active TraR Protein, a Quorum-Sensing Transcription Factor from *Agrobacterium tumefaciens*[▽]

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Received 13 October 2008/Accepted 23 March 2009

TraR of *Agrobacterium tumefaciens* is a LuxR-type quorum-sensing transcription factor that regulates genes required for replication and conjugation of the tumor-inducing (Ti) plasmid. TraR requires its cognate autoinducer *N*-3-oxooctanoyl-homoserine lactone (OOHL) for resistance of proteolysis in wild-type bacteria and for correct protein folding and solubility when overexpressed in *E. coli*. In this study, we ask whether GroESL might also play a role in TraR folding, as this molecular chaperone assists many proteins in attaining their native tertiary structure. Expression of *E. coli* GroESL in a strain expressing TraR increases the solubility of TraR and increases transcriptional activity of a TraR-dependent promoter. Both solubility and activity still require OOHL. We also studied the folding of TraR in the closely related bacterium *Sinorhizobium meliloti*. A mutation in one *groEL* gene slightly decreased the expression of a TraR-dependent promoter, strongly decreased the accumulation of TraR in Western immunoblot assays, and also strongly influenced the fate of pulse-labeled TraR.

Many bacterial proteins acquire their native tertiary structure during or shortly after translation and require no assistance from any other molecules. However, for many other proteins, folding requires or is facilitated by cytoplasmic chaperone proteins, which appear to test a variety of possible conformations of their substrates until the native conformation is found (20). We have extensively studied the folding of the quorum-sensing regulator TraR of *Agrobacterium tumefaciens*, which is a member of the LuxR family of transcription factors (25, 47, 48) and regulates genes required for vegetative replication and conjugal transfer of tumor-inducing (Ti) plasmids (18, 24, 29, 30). TraR activity requires the diffusible signal molecule *N*-3-oxooctanoyl-homoserine lactone (OOHL), which is synthesized by the TraI protein. OOHL is completely buried within the N-terminal domain of TraR, where it contributes to the hydrophobic environment of the protein core, and has virtually no contact with bulk solvent (37, 43).

We have demonstrated that TraR requires OOHL in order to fold into a stable, soluble, dimeric, protease-resistant form (22, 41, 42). Pulse-chase experiments showed that TraR can be rescued from proteolysis by OOHL only during its synthesis on ribosomes. Once synthesis is complete, apo-TraR cannot be rescued by OOHL from proteolysis (48). This finding strongly suggests that OOHL mediates the cotranslational folding of TraR and acts as an essential scaffold for TraR maturation. In the absence of OOHL, natively expressed apo-TraR was undetectable in *A. tumefaciens* by Western immunoblotting, due to its rapid proteolysis (7, 47, 48). These Western blot assays would have detected as few as one TraR molecule per cell, indicating that in the absence of OOHL, TraR is degraded extremely rapidly.

Fusion proteins containing TraR and certain other proteins are far more stable to proteolysis than is native TraR and were partially active in the absence of OOHL (6). Those foreign polypeptides of these fusion proteins were proposed to function as protein solubility enhancers and intramolecular chaperones (6). In the absence of OOHL, fused polypeptides may sequester the amino terminus of TraR from proteolysis and/or promote dimerization (6).

GroES and GroEL proteins form a complex consisting of 14 GroEL subunits and 14 GroES subunits that, together, enclose an internal chamber where protein folding occurs. GroESL is one of the major ATP-dependent cytoplasmic chaperones and is highly conserved among bacteria, archaea, and eukaryotes (2, 14, 17, 39). It interacts with a large number of unfolded or misfolded proteins and assists in their folding and remodeling during or after their synthesis. It has been proposed that in *Escherichia coli*, between 10 and 15% of the newly translated cytoplasmic proteins are associated with GroESL, and this fraction increases to about 30% under heat stress conditions (14). When the LuxR protein of *Vibrio fischeri* was expressed in *E. coli*, its solubility and activity were strongly enhanced by the overexpression of GroESL. An *E. coli groEL* mutant containing functional *luxR* and *luxICDABE* genes was only weakly luminescent (13). Overexpression of GroESL also enhanced the binding of exogenous autoinducers by LuxR (13). However, those experiments were done in a heterologous host using a high-copy plasmid, so conclusions were limited for the role of GroESL in the native strain.

Proteins orthologous to TraR and TraI were described in *Sinorhizobium meliloti* AK631 (26). The TraR protein of that strain is only 28% identical with TraR of the octopine-type Ti plasmid but regulates orthologous *tra* and *trb* genes and is inhibited by TraM (26). A mutation in a chromosomal *groEL*

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▽ Published ahead of print on 27 March 2009.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant description	Reference
Strains		
E. coli DH5α	α-Complementation	Stratagene
E. coli BL21(DE3)	*E. coli* B, P*lac*-gene 1 of bacteriophage T7	35
A. tumefaciens KYC55	Ti plasmid-less *A. tumefaciens* R10; Km^r	11
S. meliloti Rm11500	Spontaneous Sm^r mutant of AK361	26
S. meliloti Rm11501	Rm11500 containing *groELc1583*::Tn*5*	26
Plasmids		
pT-*groE*	PT7-*groESL*, ColE1; Cm^r	42
pJZ335	P*lac-traR* in pPZP201; Sp^r	44
pJZ358	PT7-*traR* in pRSERA; Amp^r	47
pJZ372	P*traI-lacZ lacI*^{q}; Tet^r	44
pJZ384	PT7-*traR* in pPZP201; Sp^r	45
pJZ410	T7 RNAP gene under P_L of bacteriophage λ; Gm^r	45
pYC335	P*lac-traR* in pPZP201; Sp^r	7
pYC337	PT7-*groESL* in pYC335; Sp^r	This study
pYC358	PT7-*groESL* in pJZ358; Amp^r	This study

gene (designated *groELc1583*::Tn*5*) abolished the production of a specific set of autoinducers whose synthesis is TraR and TraI dependent (26), suggesting that GroELc plays a positive role in this process, possibly by aiding in the folding of one of these proteins. In an elegant genetic analysis, suppressor mutations that would restore autoinducer production were sought. One such mutant had a null mutation in the *traM* gene. This protein also has an ortholog on the Ti plasmid, and both TraM proteins are TraR antiactivators (9, 10, 22, 26, 31). Apparently, the release of TraR from antiactivation somehow compensated for the loss of GroELc. One possible interpretation of these data is that TraR in this strain requires GroELc for folding and that the reduced level of TraR caused by this mutation can be compensated for by releasing it from antiactivation. It was not clear whether the original GroELc mutation completely abolished protein function, as it was caused by a transposon insertion very close to the 3′ end of the gene. It is also not known how many other *groESL* operons exist in this strain of *S. meliloti*, though *S. meliloti* Rm1021 has five *groESL* operons, two of which are on the chromosome, two of which are on pSymA, and one of which is on pSymB (1, 5, 16, 19).

The evidence that GroESL is important for quorum sensing in *S. meliloti* suggested that the same might be true in *A. tumefaciens*. Unlike *S. meliloti*, *A. tumefaciens* has only one copy of the *groESL* operon, located on the circular chromosome (21, 40). In the present study, we show that overexpression of *E. coli* GroESL enhanced TraR solubility in *E. coli* and enhanced the expression of a TraR-dependent promoter in *A. tumefaciens*. A mutation of *groELc* of *S. meliloti* impaired the accumulation and activity of *A. tumefaciens* TraR in this heterologous host. These findings all support a role for GroESL in TraR folding.

MATERIALS AND METHODS

Strains, media and reagents. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultured in LB medium at 37°C for general purposes or at 27°C for TraR overexpression. *A. tumefaciens* strains were cultured at 27°C in AT minimal medium (4). *S. meliloti* strains were cultured at 27°C in LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC) (26). Antibiotics were added at the following concentrations: spectinomycin at 100 μg per ml for *A. tumefaciens* and at 300 μg per ml for *S. meliloti*; gentamicin at 100 μg per ml for *A. tumefaciens*, at 10 μg per ml for *E. coli*, and at 50 μg per ml for *S. meliloti*; tetracycline at 2.5 μg per ml for *A. tumefaciens* and at 10 μg per ml for *S. meliloti*; ampicillin at 200 μg per ml for *E. coli*. Restriction enzymes and other DNA modification enzymes were purchased from New England Biolabs. The *N*-3-oxooctanoyl-homoserine lactone (OOHL) used in this study was generously provided by A. Eberhard and was prepared as previously described (44).

DNA manipulations. Molecular cloning and plasmid constructions were performed according to published protocols (33). Plasmid pYC358 was constructed by cutting plasmid pT7-*groESL* with SalI and HindIII and cloning a 2.5-kb fragment containing *groESL* into the same sites of plasmid pJZ358 (47). Plasmid pYC358 therefore contains both a PT7-*traR* fusion and a PT7-*groESL* fusion. Plasmid pYC337 was constructed by cloning the same 2.5-kb fragment into the corresponding sites of pYC335 (7). Plasmid pYC337 therefore contains a P*lac-traR* fusion and a PT7-*groESL* fusion. These plasmids were introduced into *A. tumefaciens* and *S. meliloti* by electroporation (4).

TraR overexpression. Strains BL21(DE3)(pJZ358) and BL21(DE3)(pYC358) were cultured at 27°C in 100 ml of LB broth containing 400 μg of ampicillin per ml and 10 μM OOHL. When cultures reached an optical density at 600 nm of 0.4, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the cultures to a final concentration of 500 μM. Incubation was continued at 27°C until the cultures reached an optical density at 600 nm of 1.0. Cells were collected, resuspended in 2 ml of TEDG buffer (8) supplemented with 100 mM NaCl, and disrupted using a French pressure minicell (15,000 lb/in^2). Total cell lysates were separated into soluble and pellet fractions by ultracentrifugation at 45,000 × *g* for 30 min. Protein samples from total, soluble, and pellet fractions were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunodetection of TraR in vivo. To compare intracellular concentrations of TraR in the wild-type *S. meliloti* strain and the *S. meliloti groELc* mutant, plasmids pJZ335 and pJZ372 were introduced into Rm11500 (wild type) and Rm11501 (*groELc1583*::Tn*5*). The resulting strains were cultured at 28°C in LB/MC medium supplemented with appropriate antibiotics, 500 μM IPTG, and 100 nM OOHL. Cells were harvested at early log phase, resuspended in 1 ml of TEDG buffer supplemented with 100 mM NaCl, and lysed using a French pressure minicell (15,000 lb/in^2). Total cell lysates were size fractionated by SDS-PAGE. Proteins were transferred by electrophoresis to a nitrocellulose membrane (Bio-Rad) and detected using preabsorbed polyclonal anti-TraR rabbit antiserum as described previously (7).

Assays of TraR-dependent activity in vivo. Bioassays of TraR activity were conducted by culturing cells either in AT minimal medium (for *A. tumefaciens* strains) or in LB/MC medium (for *S. meliloti* strains) supplemented with 500 μM IPTG (IPTG was omitted in *A. tumefaciens* cultures to reduce TraR expression) and the OOHL concentrations indicated in Fig. 2. Cells were grown with vigorous aeration for 12 h (to early log phase) at 27°C and were assayed for β-galactosidase specific activities (27). All data represent averages of at least two independent assays, and error bars represent values of the standard deviations.

Measurements of TraR turnover. Plasmids pJZ384 and pJZ410 were introduced into *S. meliloti* strains Rm11500 and Rm11501. The resulting strains were cultured at 27°C in LB/MC medium supplemented with the appropriate antibiotics and 100 nM OOHL until early log phase. Rifampin was added to the cultures to a final concentration of 200 μg per ml to block bacterial RNA polymerase activity (3). Forty minutes later, [^{35}S]methionine was added to the cultures to a final concentration of 5 μCi per ml. After an interval of 3 min, nonlabeled methionine was added to the cultures to a final concentration of 2 mM. Cells were withdrawn at various time intervals, washed with a cold Slota buffer (50 mM Tris-HCl, 0.5 M NaCl, 20 mM EDTA [pH 8.0], 0.05% Na-Sarkosyl) and ice-cold lysis buffer (50 mM Tris-HCl [pH 7.9], 200 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 1 mM β-mercaptoethanol, 0.1% phenylmethylsulfonyl fluoride, 1% NP-40, and 500 μg per ml freshly prepared lysozyme), and incubated on ice for 30 min. The resulting lysates were centrifuged at 12,000 × *g* for 3 min, and the supernatants were subjected to size fractionation by SDS-PAGE. Results were analyzed using a PhosphorImager (model 840; Molecular Dynamics).

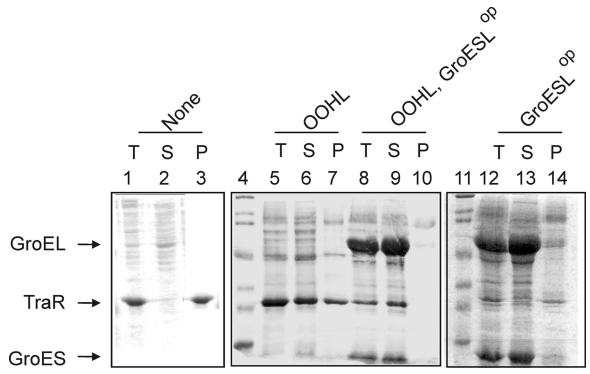

FIG. 1. Cooverexpression of TraR and *E. coli* GroESL. Strain BL21(DE3)(pJZ358) was used to detect soluble TraR in the absence of GroESL overexpression, while BL21(DE3)(pYC358) was used to detect soluble TraR in the presence of GroESL overexpression. Cells were cultured in LB medium in the presence (lanes 5 to 10) or absence (lanes 1 to 3 and lanes 12 to 14) of 10 μM OOHL. Lanes 4 and 11 contain molecular-weight standards. Letters T, S, and P represent total, soluble, and pellet fractions of the cell lysates, respectively. The gel was stained with Coomassie brilliant blue dye. GroESL^{op} refers to strains that overproduce GroESL from pYC358. This gel is representative of an experiment carried out three times with independent biological samples.

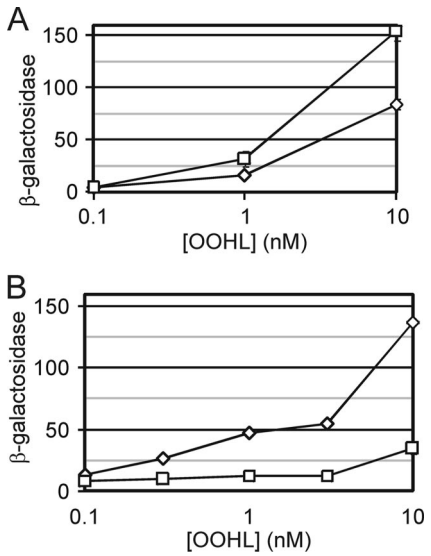

FIG. 2. (A) Overexpression of *E. coli* GroESL in *A. tumefaciens* increases TraR-dependent gene expression. Two derivatives of strain KYC55(pJZ372)(pJZ410) are compared, one containing plasmid pYC335 (diamonds), which contains the P*lac-traR* fusion only, and the other containing pYC337 (squares), which contains a P*lac-traR* fusion and a PT7-*groESL* fusion. The resulting strains were treated with OOHL at the indicated concentrations and assayed for β-galactosidase specific activity. (B) Expression of the *traI* promoter in the presence or absence of GroELc. Plasmids pJZ335 (which contains a P*lac-traR* fusion) and pJZ372 (which contains a P*traI-lacZ* reporter fusion) were introduced into Rm11500 (diamonds) and the *groELc* mutant (squares). The strains were cultured with the indicated concentrations of OOHL and assayed for β-galactosidase specific activity.

RESULTS

Cooverexpression of TraR and the *E. coli* GroESL enhances accumulation of soluble TraR. When TraR was overexpressed in *E. coli* strain BL21(DE3) using a T7 protein expression system (35) in the absence of OOHL, virtually all of the TraR protein was found in the particulate fraction, indicating that it accumulated as insoluble inclusion bodies, while the addition of OOHL to the culture medium dramatically increased TraR solubility, as approximately half of the total TraR protein was in the soluble fraction (47). We wanted to test whether overexpression of the *E. coli* GroESL proteins would further enhance the accumulation of soluble TraR. To do this, we used plasmid pYC358, which overexpresses TraR from a T7 promoter and expresses *E. coli* GroESL from a second T7 promoter on the same plasmid. As a control, we used plasmid pJZ358, which overexpresses TraR but not GroESL (47).

As observed previously (45), when TraR was overexpressed in the absence of OOHL, all detectable protein was insoluble (Fig. 1, lanes 1 to 3), while when it was expressed in medium containing 10 μM OOHL, approximately half of the total TraR protein was soluble and presumed to be folded (Fig. 1, lanes 5 to 7) (47). In contrast, when TraR and the *E. coli* GroESL were coexpressed in the presence of 10 μM OOHL, all detectable TraR protein was found in the soluble fraction (Fig. 1, lanes 8 to 10). This can best be seen by comparing lanes 7 and 10 in Fig. 1, as the former contains a large quantity of TraR, while the latter contains none. Overexpression of GroESL therefore enhanced TraR solubility, presumably by enhancing the rate at which TraR can fold. The total amount of TraR produced by these cells appears to have decreased somewhat (Fig. 1, lanes 5 and 8). It is possible that GroESL overproduction depleted the cells of the substrates for protein synthesis. Alternatively, it is possible that GroESL rescued TraR from inclusion bodies, but not from proteolysis, especially if the rescued TraR lacks OOHL and therefore is unfolded.

In the absence of OOHL, overexpression of GroESL did not cause the accumulation of soluble, stable TraR (Fig. 1, lanes 12 to 14). Furthermore, GroESL caused a drastic decrease in the total abundance of TraR (Fig. 1, lanes 1 and 12). One possible contribution to this effect could be that overproduction of GroESL may divert protein synthetic resources from TraR. We favor the alternative possibility that GroESL overproduction may rescue apo-TraR from protease-resistant inclusion bodies but that it cannot fold this protein into a mature form in the absence of OOHL. If so, then the soluble protein would be degraded by cellular proteases. We conclude, first, that GroEL enhances TraR folding and, second, that even when GroESL is overproduced, OOHL is still essential for TraR folding and protease resistance.

Overexpression of GroESL increases transcription of a TraR-dependent promoter. We also expressed TraR and *E. coli* GroESL in *A. tumefaciens* to see whether overexpression of GroESL would have any effect on TraR activity. We introduced into strain KYC55(pJZ410)(pJZ372) plasmid pYC337, which contains a PT7-*groESL* fusion and a P*lac-traR* fusion on the same plasmid, and pYC335, which contains the P*lac-traR* fusion but lacks *groESL*. Plasmid pJZ410 expresses T7 RNA polymerase while pJZ372 contains a P*traI-lacZ* fusion and *lacI*^{q} (45). In these experiments, IPTG was omitted from the broth to help limit TraR expression. Overexpression of the GroESL caused a modest, reproducible increase in the expression of the

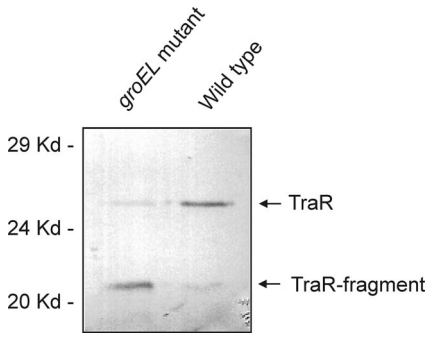

FIG. 3. Western immunoblot assays detecting TraR accumulation in the wild-type *S. meliloti* strain Rm11500 and in the *groELc* mutant Rm11501. TraR was expressed in strains Rm11500(pJZ372)(pJZ335) and Rm11501(pJZ372)(pJZ335). Accumulation of TraR was assayed as described in Materials and Methods.

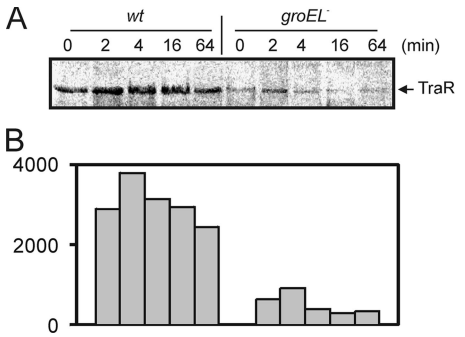

FIG. 4. Pulse-chase experiment comparing TraR stabilities in the wild-type *S. meliloti* strain Rm11500 (*wt*) and in the *groELc* mutant strain Rm11501 (*groEL*). (A) Strains Rm11500(pJZ384)(pJZ410) and Rm11501(pJZ384)(pJZ410) were used to overexpress TraR from a T7 promoter in the presence of 100 nM OOHL. [^{35}S]methionine-labeled TraR proteins were collected at various time intervals as indicated and were size fractionated by SDS-PAGE. Results were analyzed using a PhosphorImager. (B) Relative signal strength of labeled TraR shown in panel A.

traI promoter (Fig. 2A). The fact that GroESL overexpression caused only a modest enhancement may have been due to the endogenous expression of GroESL. Similarly, when GroESL was overexpressed from the weaker *lac* promoter, it did not detectably enhance expression of the reporter (data not shown). When IPTG (0.5 mM) was added into the medium to induce TraR expression from the *lac* promoter, overexpression of GroESL did not enhance expression of the target promoter (data not shown). We conclude that GroESL enhances TraR activity only when the two proteins are overexpressed at a particular ratio, as too little GroESL or too much TraR abolishes this effect.

TraR activity is defective in an *S. meliloti groELc* mutant. A previous study of *S. meliloti* (strain RM11500) has shown that mutation of the *groELc* gene blocked the activity of a TraR ortholog encoded by a conjugal plasmid of that bacterium (26). The full sequence of *groELc* is not available, and we do not know how many other homologous genes are found in that strain. However, the sequenced *S. meliloti* strain, Rm1021, has five *groESL* operons, two borne on the chromosome, two on pSymA, and one on pSymB (19). In contrast, *A. tumefaciens* has only one copy of the *groEL* genes, and despite repeated attempts, we were unable to disrupt this gene, suggesting that it may be essential. Inasmuch as *A. tumefaciens* and *S. meliloti* are closely related members of the *Rhizobiaceae* (15, 41), we decided to use the *S. meliloti groELc* mutant (Rm11501) and its isogenic parent strain (RM11500) in our study. Both strains carry an endogenous copy of *traR* on plasmid pRme41a, although it is apparently not significantly expressed under these conditions, as control strains lacking the *A. tumefaciens traR* gene did not express a TraR-dependent reporter. We introduced two plasmids into these strains: pJZ372, which has a P*traI-lacZ* fusion and *lacI*^{q}, and pJZ335, which has a P*lac-traR* fusion and expresses the native TraR protein. Both of the resulting strains grew at similar rates in LB/MC medium supplemented with the appropriate antibiotics and OOHL (data not shown). The TraR-dependent expression of the *traI* promoter was reproducibly higher in the wild-type strain than in the *groELc* mutant (Fig. 2B). We conclude that GroELc is required for maximal activity of the *A. tumefaciens* TraR and believe that residual TraR activity in the mutant may be due at least in part to GroESL proteins encoded by other *groESL* operons.

TraR accumulation in the *S. meliloti groELc* mutant. The hypothesis that GroESLc promotes TraR folding predicts that the *groELc* mutation should cause a decrease in the accumulation of TraR. Western immunoblot assays comparing TraR accumulation in Rm11500 and that in Rm11501 showed abundant levels of full-length TraR in the wild-type strain, but they also showed that only trace levels accumulated in the *groELc* mutant (Fig. 3). The mutant instead accumulated a smaller protein, the size of which is very close to the size of the N-terminal domain of TraR (residues 1 to 170). This protein was present only in trace amounts in the wild-type strain and could represent a proteolytic breakdown product of TraR. If this fragment consists of the TraR N-terminal domain, accumulation of such a protein would be predicted to inhibit the activity of the wild-type protein by the formation of inactive heterodimers (8, 28, 46).

TraR folds more effectively in the wild-type *S. meliloti* strain than in the *groELc* mutant. We performed pulse-chase experiments to directly compare TraR stability in a *groELc* mutant (Rm11501) with that in a congenic wild-type strain (Rm11500). Two plasmids were introduced into both strains, one of which (pJZ384) expresses TraR from a T7 promoter, while the other (pJZ410) contains the T7 RNA polymerase gene under the control of a P_L promoter of the bacteriophage lambda and also contains the thermosensitive *c*I857 allele of the lambda repressor. We used these two plasmids to specifically radiolabel TraR under conditions where the host RNA polymerase was inactivated using rifampin (3). Although expression of the T7 RNA polymerase was designed to be heat inducible, we previously found that transcription activity levels from the T7 promoter were similar at either 27°C or 42°C in this system (45). Therefore, we conducted all assays at 27°C. Radiolabeling of TraR was far stronger in the wild-type strain than in the *groELc* mutant (Fig. 4A), both in the presence of 100 nM OOHL. However, the TraR radiolabeled in the mutant strain had a half-life similar to that of TraR expressed in the wild-type strain. We conclude, first, that the *groELc* mutant is starved for

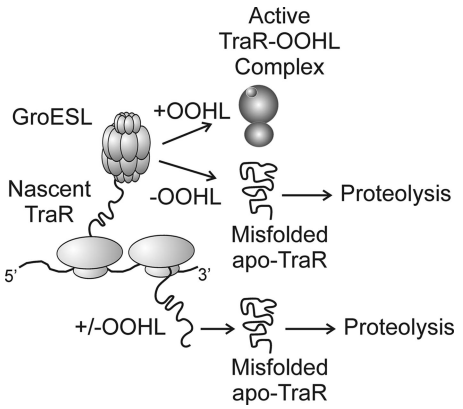

FIG. 5. A proposed model of nascent TraR folding in the absence or presence of GroESL and OOHL. TraR protein interacts with GroESL complexes during or directly after synthesis (top). In the presence of OOHL, GroESL can fold TraR monomers into soluble forms capable of dimerization and DNA binding. In the absence of OOHL, GroESL is unable to fold TraR into an active form and releases it for rapid proteolysis (middle). TraR protein that fails to interact with GroESL is targeted for proteolysis, even in the presence of OOHL (bottom).

GroEL and, second, that other copies of this gene provide low levels of functional GroEL protein. Under these conditions, the majority of TraR fails to be folded by GroESL and is rapidly degraded. A fraction of the total TraR pool is able to interact with the remaining pool of GroESL, and this TraR fraction is resistant to proteolysis, just as it is in the wild-type strain.

DISCUSSION

One of the most intriguing and puzzling properties of TraR may be its inability to accumulate and resist proteolysis in the absence of OOHL (47, 48). Most well-studied ligand binding proteins are equally stable in their ligand-free form and their ligand-bound form (12), and for such proteins, binding of ligands often causes protein conformational changes that lead to altered activity. Like TraR, several other LuxR-type proteins require acyl-homoserine lactone-type autoinducers for correct folding and protease resistance (34, 36, 38).

GroESL has been proposed to facilitate the folding of 10 to 30% of all nascent cytoplasmic proteins in *E. coli* and can also facilitate the refolding of purified proteins that have been denatured in vitro. However, at least some of these same proteins can also fold cotranslationally in vitro in the absence of GroESL. In fact, cotranslational folding of a protein in the absence of GroESL often occurs more rapidly than posttranslational folding of the same protein by GroESL. For most protein substrates, the cotranslational folding event happens within 10 to 30 s, although in other cases folding may take a much longer time (14). In general, GroESL binds to proteins that have exposed hydrophobic residues. Our data are consistent with models in which GroESL helps to protect apo-TraR from misfolding and maintains it in a state that is competent to bind OOHL, which is also essential for folding. If OOHL is absent, the protein is eventually degraded by the major cellular proteases Clp and Lon (47, 48).

The genomes of several bacterial genera encode multiple copies of GroESL (23, 32), leading to speculation that the different copies of these genes may encode proteins with unique functions (39). *S. meliloti* RM1021 bears five copies of *groESL* (1, 16, 19), and it has been proposed that each copy may have a unique biological role (26). If the same is true of strain RM15000, it might help to explain the finding that the mutation of just one of these genes causes a quorum-sensing phenotype (26). The residual level of TraR activity in the *groESLc* mutant could well be due to the other copies of *groESL*.

Our data suggest that GroESL may be just as essential for TraR maturation as is OOHL. We therefore propose a model for GroESL-dependent, OOHL-dependent cotranslational TraR folding. In this model, we consider TraR folding under two different conditions—one in the absence of OOHL (Fig. 5A) and one in the presence of OOHL (Fig. 5B). In the absence of OOHL, during translation or immediately afterwards, most or all apo-TraR proteins are bound by GroESL. However, even this chaperone cannot impart a protease-resistant conformation, and when TraR is released from the folding chamber of the chaperone, it is quickly degraded by the cytoplasmic proteases Clp and Lon (48). TraR must interact directly with OOHL and with GroESL in order to fold. It remains unclear whether GroESL and OOHL act upon TraR simultaneously or sequentially and whether OOHL binds TraR within the folding chamber of this chaperone.

ACKNOWLEDGMENTS

We thank Juan E. Gonzalez at University of Texas, Dallas, for generously providing *S. meliloti* strains Rm11500 and Rm11501, and we thank members of S.C.W.'s lab for helpful discussions and critical reviews of the manuscript during its preparation.

This work was supported by grant GM42893 from the NIH.

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