Mutations in Residues Involved in Zinc Binding in the Catalytic Site of *Escherichia coli* Threonyl-tRNA Synthetase Confer a Dominant Lethal Phenotype[▽]

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***Escherichia coli* threonyl-tRNA synthetase is a homodimeric protein that acts as both an enzyme and a regulator of gene expression: the protein aminoacylates tRNA^{Thr} isoacceptors and binds to its own mRNA, inhibiting its translation. The enzyme contains a zinc atom in its active site, which is essential for the recognition of threonine. Mutations in any of the three amino acids forming the zinc-binding site inactivate the enzyme and have a dominant negative effect on growth if the corresponding genes are placed on a multicopy plasmid. We show here that this particular property is not due to the formation of inactive heterodimers, the titration of tRNA^{Thr} by an inactive enzyme, or its misaminoacylation but is, rather, due to the regulatory function of threonyl-tRNA synthetase. Overproduction of the inactive enzyme represses the expression of the wild-type chromosomal copy of the gene to an extent incompatible with bacterial growth.**

Herman Müller first described dominant negative mutations as “antimorphs that are antagonistic mutant genes, having an effect actually contrary to that of the genes they were derived from” (17). The potential importance of such mutations in assigning gene functions was recognized a long time ago (11). Dominant negative mutations are defined as being able to inhibit the function of the wild-type gene. They have been useful in defining the DNA binding domain of phage and bacterial repressors (15) and in studying enzymes. They generally occur in multimeric enzymes and result in the synthesis of nonfunctional proteins through the assembly of a mixture of mutant and wild-type subunits (7). Dominant lethal mutations are a particular example of dominant negative mutations in which the wild-type gene product has a vital function.

In this work, we focus on a specific class of dominant lethal mutations that modify the zinc-binding site of threonyl-tRNA synthetase (ThrRS). For many aminoacyl-tRNA synthetases, the zinc atom has a structural role, shaping the enzyme in a three-dimensional structure compatible with its function (20). In ThrRS, the zinc atom is involved directly in threonine recognition by interacting with both its amino and hydroxyl groups (25, 26). The direct involvement of the zinc atom in amino acid recognition was recently shown in another case, that of seryl-tRNA synthetase from a methanogenic archaea (1). In ThrRS, the mode of threonine recognition explains how the isosteric amino acid valine is rejected by the enzyme. The zinc-binding site consists of one cysteine (C384) and two histidine residues (H385 and H511). Mutations in these amino acids confer a dominant lethal phenotype if the mutant gene is induced from the *lac* promoter on a high-copy-number plasmid (26). Because

one needs to overproduce the mutant forms of ThrRS to observe the lethal phenotype, these mutations should, strictly speaking, be called multicopy dominant lethals. Plasmids carrying these mutations were shown to be unable to complement chromosomal deletion of the gene encoding ThrRS, even under conditions where the protein is overproduced, showing that mutated enzymes are unable to carry out the aminoacylation reaction (26). This lack of activity is emphasized by the fact that mutants with very low activity are still able to complement the chromosomal deletion when overproduced to the same extent (3). The phenotypes of the mutant genes seem to be related to the absence of zinc binding since the dominant lethal phenotype is abolished in the presence of 1 mM ZnSO₄ in the growth medium (26).

Escherichia coli ThrRS has a dual function in the cell: the protein aminoacylates cognate tRNAs and is able to bind to the 5' untranslated region of its own mRNA to inhibit its translation (22). The regulatory and enzymatic functions of ThrRS are related, since the 5' untranslated region of the *thrS* gene encoding ThrRS contains two domains that are recognized similarly to the genuine anticodon arm of the tRNA^{Thr} isoacceptors (22). This dual function raises the question of the mechanism of dominance. Is dominance related to the regulatory or the enzymatic function of ThrRS or to both? The present *in vivo* and *in vitro* experimental data allow us to propose a model explaining the dominant lethal phenotype of the ThrRS zinc-binding site mutations.

MATERIALS AND METHODS

Strains and standard techniques. The *E. coli* K12 strains used are listed in Table 1. General genetic and cloning techniques were as previously described (16, 24). Lysogens carrying the fusions were selected as previously described (30). For each phage three independent lysogens were tested for β -galactosidase levels. Values varied as multiples of the lowest value, which was considered that of a monolysogen. Tet^s derivatives of strains carrying Tn10 transposons were selected as previously described (14). Oligonucleotide site-directed mutagenesis

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TABLE 1. *E. coli* and λ bacteriophage used

Strain, phage, plasmid, or construct	Genotype	Description	Source or reference
<i>E. coli</i> strains			
IBPC5311	<i>thi-1 argE3 ΔlacX74 galK2 mtl-1 xyl-5 tsx-29[?]^a rpsL recA pps</i>		30
IBPC5421	<i>thi-1 argE3 lacZ(non polar) galK2 mtl-1 xyl-5 tsx-29[?] rpsL recA1</i>		28
IBPC6183	<i>thi-1 argE3 his-4 ΔlacX74 mtl-1 xyl-5 tsx-29 supE44[?] rpsL thrSM1-9 srl-300::Tn10 recA56</i>	TetR RecA derivative of IBPC6101	5
IBPC7710	<i>thi-1 argE3 his-4 ΔlacX74 mtl-1 xyl-5 tsx-29 supE44[?] rpsL thrSM1-9 [srl-300::Tn10]^{-b} recA56</i>	TetS derivative of IBPC6183	This work
IBPC6163	<i>thi-1 argE3 his-4 ΔlacX74 mtl-1 xyl-5 tsx-29 rpsL, srl-300::Tn10 recA56</i>	TetR RecA derivative of IBPC6051	5
IBPC7502	<i>thi-1 argE3 his-4 ΔlacX74 mtl-1 xyl-5 tsx-29 rpsL, [srl-300::Tn10]⁻ recA56</i>	TetS derivative of IBPC6163	This work
IBPC7251	<i>thi-1 argE3 his-4 ΔlacX74 mtl-1 xyl-5 tsx-29 rpsL, thrSM1-9 [srl-300::Tn10]⁻ recA56</i>	TetS derivative of IBPC6183	This work
IBPC6196	<i>thi-1 argE3 his-4 ΔlacX74 mtl-1 xyl-5 tsx-29 rpsL [srl-300::Tn10]⁻ recA56</i>	TetS derivative of IBPC6163	This work
JM83R	<i>ara Δ(lac-pro) thi rpsL ϕ80dlacZΔM15 recA56</i>		5
Lambda phage			
λ MA20-10	<i>cI857 nin5 pthrS-OthrS-thrS-lacZ</i>		2
λ MA20-10ILO	<i>cI857 nin5 plac-Olac-OthrS-thrS-lacZ</i>		This work
λ MA20-10ILO-V2	<i>cI857 nin5 plac-Olac-OcthrS-thrS-lacZ</i>		This work
Plasmids			
pTet99	<i>tet</i>		3
pTetthr Δ H	<i>tet thrS</i>		3
pTetthr Δ H R583H	<i>tet thrS(R583H)</i>		3
pTetthr Δ H H385A	<i>tet thrS(H385A)</i>		26
pTetthr Δ H H385N	<i>tet thrS(H385N)</i>		26
pTetthr Δ H H385Y	<i>tet thrS(H385Y)</i>		26
pTetthr Δ H H385Y H511T	<i>tet thrS(H385Y H511T)</i>	The BsmI-BsrGI fragment of M13PhaSHthrS H511T carrying the H511T mutation was cloned between the corresponding sites of pTetthr Δ H H385Y	
pTetthr Δ H H385A R583H	<i>tet thrS(H385A R583H)</i>	The BsmI fragment of pTetthr Δ H R583H carrying the R583H mutation was cloned between the corresponding BsmI sites of pTetthr Δ H H385A	
pTetthr Δ H Tag1	<i>tet, thrS</i>	The MfeI-AscI fragment carrying the end of <i>thrS</i> was replaced by a double-strand linker carrying the His tag	
pTetthr Δ H H385A Tag1	<i>tet thrS(H385A)</i>	The end of <i>thrS</i> was replaced by the equivalent fragment from pTetthr Δ H Tag1	
pBAD30	<i>bla</i>		10
pBADthrS	<i>bla para-thrS</i>	The EcoRI-HindIII fragment of pTetthr Δ H carrying <i>thrS</i> was cloned between the same sites of pBAD30	
pMTL22			4
pBSAR1			29
pBSAR1SuUAG			29
pMTLthrU	<i>bla thrU</i>	Made by fusing the AlwNI-PstI fragment of pMTL22 to the PstI-EcoRI fragment of pBSAR1 (carrying <i>thrU</i>) and to the EcoRI-AlwNI fragment of pMTL23	
pMTLthrU(SuUAG)	<i>bla thrU(SuUAG)</i>	Made by fusing the AlwNI-PstI fragment of pMTL22 to the PstI-EcoRI fragment of pBSAR1SuUAG [carrying <i>thrU(SuUAG)</i>] and to the EcoRI-AlwNI fragment of pMTL23	
pTetthr Δ HI H385A thrU	<i>tet thrS(H385A) thrU</i>	The MluI-HindIII fragment of pMTLthrU carrying <i>thrU</i> was cloned between the AscI and HindIII sites of pTetthr Δ H H385A.	

Continued on facing page

TABLE 1—Continued

Strain, phage, plasmid, or construct	Genotype	Description	Source or reference
pTet <i>thrS</i> ΔH1 H385A <i>thrU</i> (SuUAG)	<i>tet thrS</i> (H385A) <i>thrU</i> (SuUAG)	The MluI-HindIII fragment of pMTL <i>thrU</i> (SuUAG) carrying <i>thrU</i> (SuUAG) was cloned between the <i>AscI</i> and HindIII sites of pTet <i>thrS</i> ΔH H385A	12
pTH2 pTH2SuUAG pTet <i>thrS</i> ΔH1 H385A <i>thrW</i>	<i>thrW</i> <i>thrW</i> (SuUAG) <i>tet, thrS</i> H385A <i>thrW</i>	MluI-HindIII fragment of pTH2 carrying <i>thrW</i> was cloned between the <i>AscI</i> and HindIII sites of pTet <i>thrS</i> ΔH H385A	12
pTet <i>thrS</i> ΔH1 H385A <i>thrW</i> SuUAG	<i>tet thrS</i> H385A <i>thrW</i> (SuUAG)	MluI-HindIII fragment of pTH2SuUAG carrying <i>thrW</i> (SuUAG) was cloned between the <i>AscI</i> and HindIII sites of pTet <i>thrS</i> ΔH H385A	
pTZ18R	<i>bla lacZ</i> α		Pharmacia Biochemicals, Inc.
pTZΔ20-10-V-2	<i>bla</i>	The HpaI-HindIII fragment of M13mp8Δ20-10-V-2 carrying the mutated <i>thrS</i> operator was cloned between the <i>HincII</i> and HindIII sites of pTZ18R	
M13 constructs			23
M13mp8Δ20-10ILO	<i>plac-thrS-lacZ</i> α		
M13mp8Δ20-10ILO-V2	<i>plac-thrS-lacZ</i> α	HincII-HindIII fragment from pTZΔ20-10-V-2 carrying a deleted version of the <i>thrS</i> operator replaced the wild-type fragment	
M13mp8Δ20-10-V-2	<i>thrS-lacZ</i> α	Carries the Δ(−22 to −40) deletion in the <i>thrS</i> operator (the A of the AUG is +1)	2

^a ?, marker has not been checked.

^b [*srI-300::Tn10*][−], strain has been converted to Tet sensitivity as described previously (14).

was performed on the M13mp18EX*thrS* or M13phaSH*thrS* construct using the U-containing DNA method (13). Sequencing was done on M13 phages between the cloning sites used to reintroduce the mutated restriction fragment in the tester plasmid and afterwards on the plasmid itself between the same sites.

Measurements of β-galactosidase activity. Bacteria were grown at 30°C in MOPS (morpholinepropanesulfonic acid) medium (19) supplemented with glycerol (0.4%) unless otherwise stated, all amino acids (18), tetracycline (10 μg/ml), and isopropyl-β-D-thiogalactopyranoside (IPTG) at a 1 mM concentration except when otherwise stated. β-Galactosidase levels were measured as previously described (16). In the experiment where ThrRS synthesis was induced during growth, IPTG was added at an optical density at 650 nm (OD₆₅₀) of 0.2.

Plasmid and phage constructs. The plasmid and M13 constructions are described in Table 1. Phage λMΔ20-10ILO was constructed by combining λSKS107 from *cosL* to its first HindIII site, the HindIII-EcoRI fragment of M13mp8Δ20-10ILO, and λgt4 from its EcoRI site to *cosR* as previously described (2). In this recombinant, transcription starts at the *lac* promoter through the *lac* operator and the *thrS* operator. The expression of the fusion is controlled at the transcriptional level by *lacI* and at the translational level by ThrRS. Phage λMΔ20-10ILO-V2 was constructed in the same way with M13mp8Δ20-10ILO-V2 instead of M13mp8Δ20-10ILO.

Enzyme purifications. His-tagged ThrRS carrying the H385A mutation [ThrRS(H385A)] was purified from IBPCB5421 λMΔ20-10 carrying plasmid pTet*thrS*ΔH H385A-His. A total of 500 ml of LB medium was inoculated with 5 ml of an overnight culture and grown at 30°C. At an OD₆₅₀ of 0.2, 10^{−3} M IPTG was added, and the culture was aerated for 3 h. Purification was as follows: 400 μl of nitrilotriacetic acid-nickel beads was incubated with 7 ml of the lysate in buffer A (50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 50 mM KCl, 5 mM β-mercaptoethanol) for 1 h at 4°C. The mixture was then loaded on a column, which was then successively washed with buffer A containing 0.4 M KCl and twice with buffer A containing 50 mM imidazole (to elute the chromosomal ThrRS). The mutant His-tagged ThrRS was then eluted with 500 mM imidazole in buffer A. The pooled active fractions were concentrated and dialyzed against the storage buffer (25 mM HEPES-KOH, pH 7.5, 2 mM MgCl₂, 50 mM KCl, 5 mM β-mercaptoethanol, 50% glycerol).

Footprinting experiments. Complex formation between 5′ end labeled *thrS* mRNA (15 nM) and wild-type or mutant ThrRS (0.01 to 0.5 μM) was performed at 4°C for 10 min in a reaction mixture of 25 μl containing 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 50 mM KCl, and 1 mM dithioerythritol. Hydrolysis with RNase T₁ (0.001 unit) was performed at 20°C for 5 min. Pb(II)-induced hydrolysis was done in the presence 2 μl of Pb(II) (40 mM) for 5 min at 20°C. Incubation controls were also done in parallel in order to detect nicks in the RNA. All reactions were stopped by phenol-chloroform extraction, followed by ethanol precipitation. The different RNA fragments were analyzed on a 12% polyacrylamide-8 M urea gel. For the assignment of the cleaved guanines, RNase T₁ and alkaline ladders were done in parallel.

Aminoacylation tests. Frozen pellets were resuspended in 200 μl of lysis buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, 7 mM β-mercaptoethanol) and sonicated on ice six times with 30-s pulses separated by 30-s intervals. The debris was centrifuged at 4°C for 5 min, and the supernatant was used for aminoacylation tests as previously described (5).

tRNA extraction and quantification. An overnight culture of JM83R transformed with plasmids (see Table 4 for a list) was diluted to an OD₆₅₀ of 0.2 in LB medium containing tetracycline (10 μg/ml) and grown at 37°C. At an OD₆₅₀ of 0.2, IPTG (1 mM) was added, and cells were grown for four more hours. A volume of 25 ml of culture for each strain was centrifuged, and the pellet was resuspended in 1.5 ml of buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂) and extracted with 2 ml of phenol (neutralized and saturated with Tris-HCl, pH 8) for 1 h on a vortex mixer (at medium speed). The aqueous phase was separated from the organic phase by centrifugation, and the RNA in the aqueous phase was precipitated by addition of 200 μl of 4 M NaCl and 4 ml of ethanol. After 1 h at −20°C, the precipitate was centrifuged, and the pellet was resuspended in 120 μl of 1.8 M Tris-HCl (pH 8). The dissolved pellet was incubated for 1 h at 37°C to deacylate the remaining charged tRNA. The RNA was precipitated with 480 μl of ethanol. After centrifugation, the pellet was washed twice with 1 ml of 80% ethanol and dried. The pellet was resuspended in 100 μl of water. The RNA concentration is about 50 OD₂₆₀ units/ml. Around 0.1 OD₂₆₀ unit was used for aminoacylation assays in the presence of an excess of purified ThrRS or MetRS, as previously described (5).

Expected ThrRS populations. If the total concentration of wild-type and mutant ThrRS proteins are defined as WT and M , respectively, the relative concentrations are determined as follows: for wild-type dimers, $[\text{wild type/wild type}] = \text{WT}^2/(\text{WT} + M)$; for heterodimers, $[\text{wild type/mutant}] = 2M \times \text{WT}/(\text{WT} + M)$; and for mutant dimers, $[\text{mutant/mutant}] = M^2/(\text{WT} + M)$. The value of ThrRS coming from the chromosome is assumed to be 1 for a wild-type strain without plasmid (see Table 3). For pTet*thrS*ΔH H385A R583H without IPTG (–IPTG) and pTet*thrS*ΔH H385A (–IPTG), we considered that the value for the WT is 1 and M is 1 because the mutant ThrRS proteins made by the plasmids are in concentrations too low to repress the chromosomal copy of *thrS*. For pTet*thrS*ΔH H385A R583H with IPTG (+IPTG), we considered that the value of the WT is 1 and that M is 20 because the mutant ThrRS made from the plasmid is overproduced 20-fold and does not repress the chromosomal copy of *thrS*. For pTet*thrS*ΔH H385A (+IPTG), we considered that the repression is 25-fold (see text), i.e., that the value for the WT is $1/19 \times 25$ and that M is 19.

RESULTS

The multicopy dominant phenotype of ThrRS zinc-binding site mutants is lost in a *thrS* operator-constitutive (OC) mutant. Throughout this work, we used the pTet*thrS*ΔH plasmid carrying either a wild-type or a mutated copy of the *thrS* gene expressed from the inducible *lac* promoter. This plasmid also carries the gene for the *lac* repressor and synthesizes ThrRS at about the same level as the chromosomal copy under repressed conditions and overproduces the enzyme about 20-fold when the expression of *thrS* is induced with IPTG (3). The plasmids were used to transform a *recA*-deficient strain wild-type for *thrS*. In the presence of the plasmid carrying a wild-type copy of *thrS*, the strain grows normally with (Fig. 1A, sector 6) or without (Fig. 1B, sector 6) IPTG. When the plasmid carries mutated copies of *thrS* corresponding to the H385A, H385N, and H385Y (H385 is a Zn atom binding position) changes in ThrRS, growth is normal in the absence of IPTG (Fig. 1B, sectors 7 to 9) but inhibited in its presence (Fig. 1A, sectors 7 to 9), as expected from mutants showing a multicopy dominant lethal phenotype. Equivalent results were found with changes in each of the three amino acids that bind the zinc atom (data not shown).

The multicopy dominant phenotype could be due to the repression of the wild-type chromosomal copy of the *thrS* gene. If the inactive H385A, H385N, and H385Y derivatives of ThrRS are still able to bind to the operator of the wild-type chromosomal copy of *thrS*, translation of the only active copy of the gene could be inhibited to levels incompatible with cellular growth. If this were true, the lethal multicopy dominant phenotype associated with the H385A change should be lost in the presence of another change that abolishes binding of ThrRS to the operator. We have previously shown that the R583H mutation, located in the anticodon binding domain of ThrRS, abolishes its capacity to repress the translation of its own mRNA due to its lack of affinity for the operator (3). Interestingly, the double H385A R583H mutant loses the dominant phenotype (Fig. 1A, sector 10), suggesting that the multicopy dominant phenotype is due to repression. This phenotype is expected to be lost in a strain where the chromosomal copy of *thrS* carries an OC mutation in its operator that does not allow ThrRS binding. Indeed, the pTet*thrS*ΔH derivatives carrying the H385 mutations were no longer lethal in strains where the chromosomal copy of *thrS* is expressed from a constitutive operator (Fig. 1A, sectors 2, 3, and 4).

Although these experiments indicate that the lethal pheno-

FIG. 1. Growth phenotypes of wild-type and OC *thrS* strains carrying pTet*thrS*ΔH derivatives. Strains used are IBPC 7710 (*thrSM1-11*; OC) (sectors 1 to 5) and IBPC 7502 (*thrS*⁺) (sectors 6 to 10). Plasmids used are the following: sectors 1 and 6, pTet*thrS*ΔH (wild type); sectors 2 and 7, pTet*thrS*ΔH H385A; sectors 3 and 8, pTet*thrS*ΔH H385N; sectors 4 and 9, pTet*thrS*ΔH H385Y; sectors 5 and 10, pTet*thrS*ΔH H385A R583H. The transformed strains were streaked on LB plates containing tetracycline (10 μg/ml). IPTG was either absent (B) or present at a concentration of 1 mM (A). Plates were incubated for 24 h at 37°C.

type may result from efficient repression of the expression of the chromosomal copy of *thrS* by the H385A, H385N, and H385Y mutant enzymes, alternative explanations are also possible. For instance, should wild-type/mutant heterodimers be inactive, the level of wild-type homodimeric enzyme synthesized from the chromosomal copy of *thrS* might be too low for the cell to grow. Furthermore, since the OC mutation on the chromosome causes about a 10-fold derepression of *thrS* (5), this might increase the concentration of wild-type homodimers above the minimal level required for growth, abolishing dominance. Growth in the OC chromosomal background could, a priori, also be explained if lethality were due to tRNA^{Thr} titration or misaminoacylation since the increased wild-type ThrRS concentration in the OC background would allow a more effective competition with the mutant enzymes.

ThrRS zinc-binding site mutants are able to repress the expression of the *thrS* gene during exponential growth. The repression efficiency of the mutant enzymes was measured using *thrS-lacZ* translational fusions cloned in lambda and integrated into the bacterial chromosome at *attλ*. The first set of measurements was performed under steady-state exponential growth. In the presence of a wild-type chromosomal copy of *thrS*, the *thrS-lacZ* fusion synthesizes 1,339 Miller units of β-galactosidase when wild-type *thrS* expression is not induced from plasmid pTet*thrS*ΔH (Table 2). This level dropped to 25 Miller units under induced conditions, giving a repression factor of 53. Because of the dominant lethal effects, steady-state exponential growth is not achievable with the pTet*thrS*ΔH plasmids carrying mutant versions of *thrS* under induced conditions. To circumvent this difficulty, we used the OC *thrS* mutation (Table 2). With this strain, the increased ThrRS synthesis from the OC chromosome caused a strong repression of the *thrS-lacZ* fusion independently of the plasmid, and β-galactosidase levels dropped from 1,339 to 73 Miller units in the presence of pTet*thrS*ΔH under noninduced conditions. In the presence of IPTG, the repression factor due to pTet*thrS*ΔH

TABLE 2. Effect of pTetthrSΔH and its mutated derivatives on β-galactosidase synthesized from a *thrS-lacZ* fusion

Plasmid	Effect on β-galactosidase in the indicated strain					
	IBPC7251ΔMΔ20–10 ^a			IBPC6196ΔMΔ20–10 ^b		
	Activity (Miller units) ^c		Repression ^d	Activity (Miller units) ^c		Repression ^d
	–IPTG	+IPTG		–IPTG	+IPTG	
pTetthrSΔH	73 ± 4	11.5 ± 1.1	6.3	1339 ± 25	25 ± 3	53
pTetthrSΔH H385A	78 ± 3	23 ± 1.4	3.3	1563 ± 58	ND	
pTetthrSΔH H385N	79 ± 3.6	26 ± 0.8	3.0	1631 ± 124	ND	
pTetthrSΔH H385Y	76 ± 3.6	32 ± 2.3	2.4	1667 ± 138	ND	

^a IBPC7251ΔMΔ20–10 carrying an OC *thrSM1-9* mutation corresponding to a mutation of CGU to CAU in the “anticodon” of domain 2 of the *thrS* operator. The mutation causes a strong increase (about 10-fold) of the ThrRS concentration of the cell (see text).

^b Wild-type *thrS*.

^c Activity was determined in the presence (+) or absence (–) of IPTG. All the values are averages of at least four independent measurements. The standard deviations are given. ND, not done due to lethal effects in the presence of IPTG.

^d The repression for a given plasmid is the ratio of the β-galactosidase level without IPTG to that of the level with IPTG.

was only about sixfold. With the mutant versions of *thrS*, the pTetthrSΔH derivatives caused about a threefold repression, somewhat lower than the wild-type ThrRS. Previous experiments (26) showed that these pTetthrSΔH derivatives overproduce the mutant enzymes to about the same level as the wild-type ThrRS (Fig. 2C).

When overproduced, ThrRS zinc-binding site mutants repress the expression of the *thrS* gene. Because the level of repression caused by ThrRS H385A, H385N, or H385Y mutants during steady-state exponential growth was quite low, we also measured *thrS* expression from a *thrS-lacZ* fusion under non steady-state conditions, i.e., after induction of the synthesis of the ThrRS mutants and before cellular growth stops due to their dominant lethal effect. In this experiment, the *thrS-lacZ* translational fusions were expressed from the inducible *lac* promoter instead of the constitutive *thrS* promoter. We induced both the synthesis of the mutant ThrRS proteins and the fusion simultaneously at an OD₆₅₀ of 0.2. After induction, β-galactosidase measurements were made for about a generation while growth was still normal. In the presence of the empty pTet99 vector, β-galactosidase levels increased, showing the time course of synthesis of the ThrRS-β-galactosidase hybrid (Fig. 2A). In the presence of pTetthrSΔH H385A or pTetthrSΔH H385N, β-galactosidase levels were considerably lower, albeit not quite as low as in the presence of plasmid pTetthrSΔH carrying wild-type *thrS* (Fig. 2A). A similar result was found with the H385Y H511T mutant. This double replacement is predicted to completely fill the cavity left empty by the absence of the zinc due to the H385Y change and to cause minimal local distortion (26). With a *thrS-lacZ* fusion carrying a constitutive *thrS* operator, β-galactosidase levels increased in the presence of both pTetthrSΔH and pTetthrSΔH H385A plasmids (Fig. 2B), as expected for a fusion that is insensitive to ThrRS levels. The concentration of ThrRS overproduced by the different plasmids was shown to be equal in all experimental conditions (Fig. 2C). These data strongly suggest that the mutant proteins are able to repress the expression of *thrS*, albeit about twofold less efficiently than the wild-type enzyme. Although we have no clear explanation for this twofold difference in repression between the wild-type and mutant ThrRS proteins, this factor is taken into account in the rest of this work as discussed below.

Amino acid changes in the zinc-binding site only marginally affect ThrRS binding affinity to its own mRNA. If mutations in the zinc-binding site only marginally affect the capacity of ThrRS to repress the translation of its own mRNA, the corresponding mutant proteins should bind to this mRNA with an affinity approaching the wild-type enzyme. Wild-type and H385A His-tagged proteins were purified in parallel. The wild-type enzyme was purified from a strain containing a deletion of the chromosomal copy of *thrS* and transformed with the plasmid pTetthrSΔH Tag1 while the H385A mutant enzyme was purified from a strain transformed with plasmid pTetthrSΔH H385 Tag1 and carrying the wild-type chromosomal *thrS* allele, since the mutant enzyme is unable to complement a strain deleted of *thrS*. The quantity of wild-type ThrRS was minimized by adequate induction conditions (see Materials and Methods) and because the H385 Tag1 ThrRS synthesized from the multicopy plasmid represses the synthesis of the wild-type enzyme from the unique chromosomal copy of *thrS* after induction. Also, the purification protocol was adapted to prevent contamination by the homodimeric wild-type enzyme (see Materials and Methods). The contamination of the H385A His-tagged ThrRS by the wild-type enzyme was kept to a minimum since we were not able to identify a significant signal of for the wild-type ThrRS by electrospray mass spectrometry (data not shown).

Footprinting assays were used to compare the capacity of H385A and wild-type ThrRS to protect the *thrS* operator from enzymatic and chemical hydrolysis. We used two single-stranded specific probes, RNase T₁ (specific for unpaired G) and Pb-induced cleavages (Fig. 3). As previously shown, most of the RNase T₁ cleavages and Pb-dependent cleavages occur in single-stranded regions, mainly in the apical loops of domains 2 and 4 and in domain 3 (21). Several Pb-induced cleavages were also observed in the internal loop of domain 2 and at the bulged uridine –81 in domain 4. The H385A enzyme caused strong protections against RNase T₁ and Pb(II) in the same regions of the mRNA as the wild-type ThrRS (Fig. 3). Moreover, the mutant enzyme produced an equivalent footprint to the wild-type enzyme over the same concentration range. These data indicate that the mutant enzyme binds the operator region with a very similar affinity to the wild-type enzyme and correlates well with the in vivo data showing that

FIG. 2. Repression by H385 ThrRS mutants with wild-type and OC *thrS-lacZ* fusions expressed from the *lac* promoter. β -Galactosidase measurements (Miller units) were made every 10 min for 2 h (B) or every 30 min for 3 h (A) after induction. (A) IBPC5421 (*thrS*⁺) monolysogenized with phage λΔ20-10ILO carrying a *thrS-lacZ* translational fusion expressed from the *lac* promoter and operator and carrying the wild-type *thrS* operator was transformed with pTet99 (□), pTetthrSΔH H385Y H511T (+), pTetthrSΔH H385A (◆), pTetthrSΔH H385N (X), and pTetthrSΔH (■). (B) IBPC5421 (*thrS*⁺) monolysogenized with λΔ20-10ILO was transformed with pTetthrSΔH (■) or pTetthrSΔH H385A (□); IBPC5421 (*thrS*⁺) monolysogenized with λΔ20-10ILO-V2 was transformed with pTetthrSΔH (◆) or pTetthrSΔH H385A (X). Phage λΔ20-10ILO-V2 has the same structure as λΔ20-10ILO but carries an OC *thrS* operator. (C) Western blotting with the extracts from cultures of panels A and B spun down at an OD₆₅₀ of 0.35. Western blotting was performed with bacteria at an OD₆₅₀ of 0.025. IBPC5421 monolysogenized with λΔ20-10ILO was transformed with pTet99 (lane 1), pTetthrSΔH (lane 2), pTetthrSΔH H385A (lane 3), pTetthrSΔH H385N (lane 4), pTetthrSΔH H385Y H511T (lane 5), pTetthrSΔH (lane 6), or H385A (lane 7); IBPC5421 monolysogenized with λΔ20-10ILO-V2 was transformed with pTetthrSΔH (lane 8) or pTetthrSΔH H385A (lane 9).

ThrRS H385A is able to repress the expression of the *thrS* chromosomal gene at about the same level as the wild-type enzyme. As we have previously shown, mutations in ThrRS that decrease regulation by a twofold factor had only a slight effect on mRNA binding (3).

A decrease in ThrRS level to less than 25% of wild-type blocks bacterial growth. Under exponential growth in an OC *thrS* mutant background, pTetthrSΔH repressed the expression of *thrS* sixfold and pTetthrSΔH carrying H385A, H385N, or H385Y repressed *thrS* expression about threefold, i.e., about half as efficiently (Table 2). The same twofold difference between wild-type and H385 A and N mutants is found in the experiment shown in Fig. 2, under non-steady-state conditions where ThrRS synthesis is induced. During steady-state exponential growth, the induced pTetthrSΔH plasmid encoding wild-type ThrRS causes a 53-fold repression. We thus suspect that, if steady-state exponential growth could be reached with the fully induced H385 ThrRS mutants, repression of the wild-type chromosomal copy of *thrS* would also be about half of the wild-type enzyme, i.e., about 25-fold. The question is whether or not such a 25-fold decrease in wild-type ThrRS levels is sufficient to stop bacterial growth and hence to explain the dominant lethal effects of the overproduced ThrRS mutants.

To answer this question, we constructed a plasmid where *thrS* was expressed from the inducible arabinose promoter. This plasmid was used to transform a strain with a deletion in the chromosomal copy of *thrS* in such a way that the plasmid is the sole source of ThrRS. In the presence of 0.2% arabinose (the *araBAD* promoter is fully induced), the strain grew with the same doubling time as the wild-type strain, which expresses *thrS* from the chromosome (Fig. 4A). Upon withdrawal of arabinose (which stops *araBAD* promoter activity) at an OD₆₅₀ of 0.01, growth proceeded for several hours at an unchanged rate. At an OD₆₅₀ of about 0.2, growth clearly leveled off and stopped at 0.45. Thus, the strain can grow normally for a little more than five doublings on the quantity of ThrRS accumulated during growth in the presence of arabinose. We measured the ThrRS activity levels after growth had stopped. A wild-type strain, isogenic to that with the chromosomal copy of *thrS* deleted, synthesized 2,605 units of ThrRS as measured by the aminoacylation assay, where 1 unit is 1 pmol of tRNA^{Thr} aminoacylated per min per mg of protein (Fig. 4B). The level of ThrRS in the arrested deleted strain was only 620 units, i.e., about four times lower. It thus seems that the minimum ThrRS level required for growth is about 25% of the wild-type level under these conditions. The 25-fold repression of the chromosomal copy of *thrS* due to the presence of pTetthrSΔH H385A would thus be sufficient to explain the dominant effect.

ThrRS wild-type/mutant heterodimers are capable of aminoacylation. The multicopy dominant lethal phenotype could also have been due to the lack of activity of wild-type/mutant heterodimers. We assume that the H385A and R583H mutations have no effect on ThrRS dimerization for the following reasons: (i) both mutations are far from the subunit interface, and (ii) they do not cause any global effects since both mutant proteins are stable and autoregulation is almost normal for H385A.

To determine whether wild-type/mutant heterodimers could aminoacylate tRNA^{Thr}, we set the total ThrRS quantity synthesized from the strain that produces only the chromosomal enzyme to the arbitrary value of 1. Previous results have shown that pTetthrSΔH plasmid derivatives synthesize ThrRS at about the same level as the chromosome under noninducing conditions (3), i.e., at a relative level of 1. We first considered the case of pTetthrSΔH H385A R583H, which synthesizes an

FIG. 3. Affinity of wild-type and H385A ThrRS for the *thrS* operator as measured by footprinting experiments. The secondary structure of 5' end labeled *thrS* operator was probed either with Pb(II) (A) or with RNase T₁ (B) in the presence and absence of wild-type (WT) and H385A His-tagged synthetases as described in Materials and Methods. Concentrations of wild-type His-tagged ThrRS added were 0.01, 0.05, 0.1, and 0.5 μM. Concentrations of His-tagged H385A ThrRS added were 0.05, 0.1, and 0.5 μM. (C) Secondary structure model showing the regions protected by ThrRS. Empty arrow, RNase T₁ cleavage; filled arrow, Pb(II)-induced cleavage; black point, protection induced by ThrRS binding; SD, Shine-Dalgarno sequence.

inactive enzyme and an inactive regulator (see above). A strain carrying both the chromosomal copy of *thrS* and the noninduced pTet*thrS*ΔH H385A R583H should produce ThrRS at a relative quantity of 2. The concentrations for the different molecular species relative to the wild-type ThrRS would then be expected to be 0.5 for the two homodimeric species and 1 for wild-type/mutant heterodimers (Table 3, -IPTG). In this case, the relative concentration of wild-type/wild-type homodimers (0.5) is larger than the lower limit essential for growth (0.25) (Fig. 4). When ThrRS mutant synthesis is induced from the plasmid, the chromosomal copy of *thrS* still synthesizes a relative quantity of 1 since the mutated ThrRS made from the plasmid is incapable of repression. Under the same conditions, pTet*thrS*ΔH derivatives overproduce ThrRS about 20-fold (3). Therefore, the total quantity of ThrRS made by a strain carrying the induced pTet*thrS*ΔH H385A R583H plasmid is expected to be 21. Under these conditions, the expected concentrations of the wild-type homodimer is only 0.05 (Table 3, +IPTG). Since strains transformed with pTet*thrS*ΔH H385A R583H can grow under induction conditions, where does the active ThrRS come from? Growth cannot be due to the dimeric mutant population since induced pTet*thrS*ΔH H385A is unable to complement a chromosomal deletion of *thrS* (26). The concentration of the wild-type homodimeric ThrRS is also under the threshold concentration of 0.25 that stops growth (Fig. 4). Therefore, the data indicate that the growth must be assumed by a heterodimeric ThrRS population that is active.

We then turned our attention to the case of pTet*thrS*ΔH H385A under induced conditions (Table 3, +IPTG). Our experiments indicate that wild-type ThrRS (synthesized from pTet*thrS*ΔH) represses chromosomal *thrS* expression 50-fold under induced conditions in steady-state exponential growth (Table 2). Since the level of repression due to pTet*thrS*ΔH H385A is about twofold lower (Table 2 and Fig. 2), it is reasonable to assume that ThrRS(H385A) represses the wild-type chromosomal copy of *thrS* at least 25-fold if steady-state exponential growth could be achieved (see above). Under these circumstances, we would expect the relative concentration of wild-type homodimers to be extremely low (Table 3, +IPTG), that of the mutant homodimers to be almost equal to 20 and that of the active heterodimers to be 0.004. Neither the mutant homodimers nor the wild-type homodimers (the latter being present at negligible quantities within the cell) could sustain growth. The heterodimers would be present at about 0.004 of the wild-type level, which is clearly not enough to sustain growth even if they are active. When pTet*thrS*ΔH H385A is induced, we would expect growth to stop when the level of active heterodimers decreases under the minimal quantity of active ThrRS, i.e., about 25% of the wild-type concentration.

Lethality cannot be explained by tRNA^{Thr} titration or misaminoacylation. Another potential explanation for the lethality of the induced pTet*thrS*ΔH H385A plasmid was the sequestration of tRNA^{Thr} by the overproduced inactive enzyme. To test this hypothesis, we cloned the *thrU* or *thrW* genes encoding two different isoacceptors of tRNA^{Thr} into the pTet*thrS*ΔH H385A

FIG. 4. Growth curves and ThrRS activity when ThrRS is in limiting quantities. Strains were grown in MOPS-glycerol supplemented with amino acids and tetracycline as described in Materials and Methods. Overnight precultures were grown with 0.2% arabinose, centrifuged, washed twice with 10^{-2} M MgSO_4 , and resuspended (at an OD_{650} of 0.01) in the same growth medium either in the presence of 0.2% Ara ($\square$, IBPC6881 pBADthrS) or in its absence ($\blacksquare$, IBPC6881 pBADthrS; $\blacklozenge$, IBPC5311). Results of aminoacylation tests are shown below the graph. Bacteria at an OD_{650} of 0.5 were taken from the cultures at different times (IBPC6881 pBADthrS with arabinose, 545 min; IBPC6881 pBADthrS without arabinose, 765 min; IBPC5311, 606 min). Samples were frozen at -80°C and used for aminoacylation assays as described in Materials and Methods.

plasmid to overproduce each of these isoacceptors from the same plasmid as ThrRS. As a control, we cloned the genes for nonsense suppressor derivatives of the two tRNAs. These suppressor derivatives of tRNA^{Thr} are known to be aminoacylated only at extremely low levels (29). The pTetthrSΔH H385A thrU and pTetthrSΔH H385A thrW plasmids overproduce aminoacylatable tRNA^{Thr} about sixfold compared to the pTetthrSΔH H385A thrU(SuUAG) and pTetthrSΔH H385A thrW(SuUAG) controls (Table 4). We determined whether overproduction of tRNA^{Thr} had an effect on dominance by testing growth (on LB plates supplemented with tetracycline at 10 $\mu\text{g}/\text{ml}$) of a strain (JM83R) transformed by this set of plasmids in the presence and absence of IPTG at different concentrations. We never observed an increase in the colony size in the presence of an excess of aminoacylatable tRNA^{Thr} compared to the situation in the presence of the nonaminoacylatable derivative (data not shown). In other words, the overproduction of tRNA^{Thr} never caused a decrease of the multicopy dominant phenotype. Therefore, tRNA^{Thr} sequestration does not seem to explain this phenomenon.

A final potential explanation of multicopy dominance was misaminoacylation of tRNA^{Thr} by closely related amino acids such as serine and valine. The wild-type enzyme uses two different mechanisms to prevent misaminoacylation of tRNA^{Thr} (6). Through the zinc ion binding site, ThrRS can

discriminate threonine from valine, while the N-terminal domain can hydrolyze Ser-tRNA^{Thr}. The purified ThrRS(H385A) did not significantly aminoacylate tRNA^{Thr} with valine or serine compared to the wild-type enzyme, indicating that misaminoacylation most probably does not explain the lethal dominance (data not shown).

DISCUSSION

The crystal structure of *E. coli* ThrRS revealed an unforeseen feature: the existence of an essential zinc ion in the catalytic site. The cysteine and the two histidines involved in zinc binding are conserved throughout evolution. The zinc ion is directly involved in threonine recognition, forming a pentacoordinate intermediate with both the amino group and the side chain hydroxyl, and prevents the activation of the isosteric valine. To assess the role of the catalytic zinc, the three residues interacting with the metal (C334, H385, and H511) were mutated independently. The effects were first measured by investigating the capacity of the mutant genes to complement a strain with an inactivated chromosomal copy of *thrS*, the gene encoding ThrRS. All three changes completely abolished complementation under both induction and noninduction conditions (26). These data indicate that the aminoacylation activity is strongly affected (see also above). If overproduced from a multicopy plasmid, the ThrRS C334, H385, and H511 mutants cause a dominant lethal phenotype in a wild-type strain; i.e., they inhibit bacterial growth even in the presence of a wild-type copy of the *thrS* gene. The dual function of ThrRS as a regulator and an enzyme raised the possibility that the lethal dominance phenotype was related to one or both of these functions.

The present work indicates that the multicopy dominance of the H385 mutation is solely explained by its regulatory function. The evidence relies on independent sets of experiments. We first showed that, when overproduced, the H385A mutant represses the expression of the chromosomal copy of *thrS* to a level too low to permit growth. We also investigated different ways with which the H385A mutant could interfere with the wild-type enzymatic function. Our data do not support the idea that dominance could be due to tRNA^{Thr} titration or misaminoacylation by the mutant enzyme. The fact that the substitution of only one amino acid interacting with zinc drastically affects aminoacylation but does not strongly affect mRNA binding supports the idea that the coordinated zinc has a specific catalytic role rather than a global structural one. Previous experiments also indicated that the catalytic site of the enzyme is not required for efficient mRNA binding (3). In addition, the

TABLE 3. Expected ThrRS populations

Plasmid	IPTG	Expected ThrRS concn relative to the single-copy chromosomal situation ^a		
		WT/WT	WT/MUT	MUT/MUT
pTetthrSΔH H385A R583H	—	0.5	1	0.5
pTetthrSΔH H385A R583H	+	0.05	1.9	19.05
pTetthrSΔH H385A	—	0.5	1	0.5
pTetthrSΔH H385A	+	1/4,512,500	0.004	19.996

^a WT, wild type; MUT, mutant. Calculations are explained in Materials and Methods.

TABLE 4. The effect of tRNA overproduction on dominance

Plasmid	Growth		Level of tRNA (pmoles/OD ₂₆₀) ^a		
	–IPTG	+IPTG	tRNA ^{Thr}	tRNA ^{Met}	tRNA ^{Thr} /tRNA ^{Met}
pTet <i>thrS</i> ΔH H385A	+	–	39.4	40.6	0.97
pTet <i>thrS</i> ΔHI H385A <i>thrU</i>	+	–	145.6	33.7	4.3
pTet <i>thrS</i> ΔHI H385A <i>thru</i> (SuUAG)	+	–	24	35.8	0.67
pTet <i>thrS</i> ΔHI H385A <i>thrW</i>	+	–	195.6	34.8	5.62
pTet <i>thrS</i> ΔHI H385A <i>thrW</i> (SuUAG)	+	–	24.4	25.8	0.94

^a tRNA^{Thr} levels in the cells were determined after RNA extraction and aminoacylation as described in Materials and Methods. tRNA^{Met} levels were also measured and used as standard for RNA extraction efficiency.

crystal structure of the operator domain 2 associated with the enzyme (31) strongly suggests that the catalytic domain of ThrRS is located far away from the operator, as indicated by the three-dimensional model of the ThrRS-mRNA complex derived from extensive genetic, biochemical, and structural data (3).

Finally, we provide evidence that wild-type/H385A mutant heterodimers are still capable of aminoacylation despite the fact that H385A abolishes enzymatic activity and that R583H strongly affects the binding of the anticodon of tRNA^{Thr}, the major identity element for this tRNA. This indicates that the function of the catalytic site of the wild-type subunit is independent of that of the other subunit at least to the extent tested here.

In addition to the zinc-binding site mutants, we have previously isolated other kinds of dominant lethal mutations in ThrRS. For instance, we have characterized a superrepressor that strongly represses *thrS* expression and binds to the operator with a 100-fold increased affinity compared to the wild-type enzyme (3). If cloned into pTet99, the plasmid vector used in this study, the corresponding mutant allele, *thrS4118* (previously named *thrS4-11-8*), is lethal in the presence of IPTG at 1 mM, i.e., under fully induced conditions. In contrast to the zinc-binding site mutants, ThrRS4118 aminoacylates the tRNA^{Thr} similarly to the wild-type enzyme (3). Interestingly, this mutant, if overproduced allows bacterial growth when the chromosomal wild-type copy of *thrS* is deleted; i.e., the mutant is lethal only in the presence of the wild-type copy of *thrS* (M. Graffe, unpublished data).

ThrRS is, by no means, the only repressor in the translational machinery in *E. coli*. The most representative group consists of the control ribosomal proteins, which, in general, are primary rRNA binding proteins that have the ability to recognize their own mRNA and inhibit their translation (32). One might think that a mutant regulatory ribosomal protein that binds normally to its operator but that is not able to participate in assembly should have a dominant lethal phenotype. In fact, such a mutant has been characterized in our laboratory in ribosomal protein L20, which regulates the translation the *rpmI-rplT* operon encoding ribosomal proteins L35 and L20 itself (8). Ribosomal protein L20 is composed of two domains, a globular domain that recognizes the helix 40-helix 41 junction of 23S rRNA and a long α -helical N-terminal extension that contacts several quite distant ribosomal rRNA sites in domains I and II of the 23S (27). We have isolated a deletion in the gene for ribosomal protein L20 that yields a

protein without its α -helical N-terminal extension (9). If cloned on a multicopy plasmid under control of the *lac* regulatory regions, this mutant allele gives a dominant lethal phenotype under induction conditions. We have shown that this L20 derivative recognizes its own mRNA as the wild-type protein in vitro and that it is able to repress the translation of its own mRNA in vivo (9). We therefore believe that this mutant is able to perform regulation but not assembly.

These dominant lethal mutants are powerful genetic tools to investigate a particularly interesting property of these translational repressors, namely, the relationship between their primary biological role, aminoacylation for ThrRS and ribosome assembly for L20, and the way they regulate the translation of their own mRNAs.

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