Hypomodification of the Wobble Base in tRNA^{Glu}, tRNA^{Lys}, and tRNA^{Gln} Suppresses the Temperature-Sensitive Phenotype Caused by Mutant Release Factor 1[▽]

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In *Escherichia coli*, release factor 1 (RF1) is one of two RFs that mediate termination; it specifically recognizes UAA and UAG stop codons. A mutant allele, *prfA1*, coding for an RF1 that causes temperature-sensitive (Ts) growth at 42°C, was used to select for temperature-resistant (Ts⁺) suppressors. This study describes one such suppressor that is the result of an *IS10* insertion into the *cysB* gene, giving a Cys[−] phenotype. CysB is a transcription factor regulating the *cys* regulon, mainly as an activator, which explains the Cys[−] phenotype. We have found that suppression is a consequence of the lost ability to donate sulfur to enzymes involved in the synthesis of thiolated nucleosides. From genetic analyses we conclude that it is the lack of the 5-methylaminomethyl-2-thiouridine (mnm⁵s²U) modification of the wobble base of tRNA^{Glu}, tRNA^{Lys}, and/or tRNA^{Gln} that causes the suppressor phenotype.

During protein synthesis, the codons in the aminoacyl site of the ribosome are read either by an aminoacyl-tRNA or by a release factor (RF). The outcome of these two processes is either elongation of the growing peptide or termination of elongation.

tRNAs from all organisms contain modified nucleoside derivatives of the four common nucleosides (21), and tRNA thiolation is a frequent modification. In bacteria the uridine in the wobble position in tRNA^{Lys}, tRNA^{Glu}, and tRNA^{Gln} is modified to 5-methylaminomethyl-2-thiouridine (mnm⁵s²U) (Fig. 1) (2). The sulfur in the thiolated nucleoside originates from cysteine, and the IscS enzyme is required to initiate the transfer from cysteine to the different tRNA nucleosides (19, 25). Cysteine is transferred either to the [Fe-S]-independent enzymes ThiI and TusA or to the [Fe-S]-dependent enzyme IscU (Fig. 2) (20).

There are two class 1 RFs with different specificities in bacteria: RF1 recognizes stop codons UAG and UAA, while RF2 terminates at UGA and UAA. RF3 (a class 2 RF) releases RF1 and RF2 from the ribosome in a GTP-dependent manner after the completed protein has been released (15).

RF1 is encoded by the *prfA* gene, and the *prfA1* allele codes for a factor that causes a temperature-sensitive (Ts) growth phenotype at 42°C (28, 29). In this study, we have mapped the position of an extragenic suppressor of the Ts phenotype. This suppressor mutation is linked to an *IS10* insertion into the *cysB* gene. CysB is a transcription factor involved in the regulation of the cysteine regulon (18). Lack of CysB protein makes a strain cysteine dependent on M9 minimal medium. Genetic analyses have shown that the suppressor phenotype is caused

by hypomodification of mnm⁵s²U in the wobble position of tRNAs reading NA(A/G) codons.

MATERIALS AND METHODS

Media, strains, and plasmids. The strains used in this study are listed in Table 1. Media and genetic procedures were those described by Miller (23). Where appropriate, the media contained 25 µg/ml tetracycline (Tc), 200 µg/ml ampicillin, 50 µg/ml kanamycin, or 20 µg/ml chloramphenicol (Cm). Unless otherwise stated, cysteine and methionine were added to final concentrations of 100 µM and 200 µM, respectively (24).

Construction of strains. A mini-Tn10 pool in MG1655 was isolated using the system described by Kleckner et al. (17).

To construct strains IIG1 and IIG2, a P1 lysate grown on strain MRA532 was used to transduce AD16, with selection for Tc^r. Transductants were scored for slow/fast growth and ability to grow on M9 minimal plates. One fast-growing isolate, which grew on M9 minimal medium without cysteine, was named IIG1, and one slow-growing clone, which could not grow without cysteine, was named IIG2.

Construction of plasmid pIIG1. Procedures for plasmid construction and transformation were those described by Sambrook et al. (30). A piece of the *cysB* gene covering the insertion site for *IS10* was amplified from MG1655 using primers CysB1 (5′-TAAATCGTATTAGTCACCCGCCAGG-3′) and GI42ned (5′-CAGCCCTAACCGGACGTAAGTTTTA-3′). The amplified fragment was cloned into the pDrive vector (Qiagen), and the sequence was confirmed.

PCR and sequence analysis. Inverted PCR was performed as described by Higashitani et al. (12).

PCR was done using Ready-To-Go PCR beads (GE Healthcare). Sequence analysis was done by Eurofins MWG Operon (Germany).

β-Galactosidase assay. Bacteria were grown at 37°C in M9 minimal medium supplemented with all 20 amino acids (24). The assay was performed as described in reference 34.

RESULTS

Map position of the suppressor mutation. To increase knowledge about the function of RF1 and the termination process, we have studied a mutant allele of RF1 that causes a temperature-sensitive growth phenotype (29). This mutant was used to isolate a collection of independent Ts⁺ suppressor mutants at 42°C (14). One of these suppressor mutants, strain MRA475, was found not to grow on M9 minimal plates. This feature was used to isolate a mini-Tn10 linked to the suppress-

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FIG. 1. Structure of $\text{mnm}^5\text{s}^2\text{U}$.

sor locus. A P1 lysate grown on a mini-Tn10 pool (see Materials and Methods) was transduced to strain MRA475, with selection for growth on M9 minimal plates supplemented with Tc. The few transductants obtained were found to be Ts, presumably as a consequence of loss of the suppressor mutation. One clone, MRA533, was kept for further analysis. The frequency of cotransduction with the suppressor was found to be about 0.8, which corresponds to a distance of approximately 0.15 min (33). To determine the position of Tn10, inverted PCR was done from the ends of the transposon, and the resulting products were sequenced. It was found that Tn10 was inserted into the *yciS* open reading frame. About 0.15 min away from *yciS* is the *cysB* gene, and since strain MRA475 cannot grow on minimal medium, *cysB* was a potential locus for the suppressor mutation. Addition of cysteine to M9 minimal plates enabled strain MRA475 to grow. The *cysB* gene codes for a transcription factor for the *cys* regulon (18). To determine the precise position of the suppressor mutation, the region covering *topA-cysB* was sequenced, and an IS10 element was found in the *cysB* sequence, with nucleotides 261 to 269 duplicated as an insertion site. The insertion probably leads to loss of expression of the CysB protein.

Genetic evidence that lack of CysB causes the suppressor phenotype. To address the question whether the observed suppressor phenotype is due to a loss of CysB activity, strain MRA475 was transformed with a plasmid-borne wild-type copy of the *cysB* gene, pCa24N, strain JW1267 (16). The transformants were tested for growth at 37°C and 42°C. As expected, they grew fast at 37°C and had regained the Ts phenotype, which indicates that loss of the CysB protein is responsible for the suppressor phenotype (data not shown).

To verify that the insertion of IS10 causes the suppressor phenotype, a marker rescue experiment was performed. A fragment of the wild-type *cysB* gene (788 bp) was amplified by using primers flanking the region where the IS10 is inserted. The fragment was cloned, creating plasmid pIIG1, which was transformed into strain MRA475. The transformed strain was plated onto M9 minimal plates with ampicillin to select for a Cys⁺ phenotype. Transformants were tested for growth at 37°C and 42°C and were found to be Ts. If the insertion of IS10 causes the suppressor phenotype, the Ts phenotype can be restored only by homologous recombination between the chromosome and the fragment on the plasmid. A Ts clone was

FIG. 2. Model for sulfur trafficking in the synthesis of thiolated nucleosides in *Salmonella enterica* serovar Typhimurium. IscS gets the sulfur from cysteine and transfers it to ThiI, TusA, or IscU. IscU transfers the sulfur to TtcA and MiaB, which synthesize s^2C and ms^2 , respectively. ThiI donates its sulfur to s^4U , while the transfer of sulfur from TusA to s^2U goes via several enzymes.

sequenced, and indeed, we found that recombination had taken place and the IS10 was lost from the chromosome.

Next, we combined the *prfA1* mutation with an inactivated *cysB* gene to confirm the suppressor phenotype. To do this, we used strain JWK1267 from the Keio collection, carrying the *cysB* gene replaced by a Cm^r cassette (4), and transduced it using a phage lysate grown on strain MRA7 (*prfA1*), selecting for Tc^r . Strain JWK1267 (ΔcysB) grows slowly, but faster than strain MRA475 (*prfA1 cysB::IS10*), on Luria broth agar (LA) plates. The transductants were tested for growth at 42°C, and two classes were found in accordance with the coupling frequency between *prfA1* and Tn10. One class grew like the recipient both at 37°C and at 42°C, whereas the other class grew like MRA475 at both temperatures, i.e., more slowly than JWK1267. We hypothesize that only Tn10 was transduced in the former class, while both Tn10 and *prfA1* were transduced in the second class. This result was verified by transduction of Tn10 from each class to MG1655 with selection for Tc^r . As expected, the first class yielded only Ts⁺ transductants, while the other class yielded Ts transductants with a 40% coupling frequency to Tn10. Hence, we conclude that lack of the CysB protein does suppress the Ts phenotype caused by *prfA1*.

Does CysB affect regulation of the gene for RF1? CysB is a transcription factor for the *cys* regulon; it functions as an activator of most operons but is an autoregulatory repressor (18). It was shown previously that overexpression of mutant RF1 suppresses the Ts phenotype (28). Therefore, we investigated whether the CysB protein also represses the gene for RF1. The *prfA* gene is the second gene in an operon, preceded by the *hemA* gene, and there are 41 nucleotides between the two genes, which allows for regulatory elements. Therefore, to test our hypothesis, we used strain AD16, which carries the beginning of the *hemA* operon, starting from the promoters in front of the *hemA* gene up to the beginning of the *prfA1* gene, fused to *lacZ*. Such a construct measures the expression of RF1 both transcriptionally and translationally (9). *cysB::IS10* was transduced to strain AD16, creating strains IIG1 and IIG2.

β -Galactosidase activity was measured in strains IIG1 and IIG2. The results showed that expression in the two strains was approximately the same (5.8×10^{-3} and 4.8×10^{-3} U, respectively). We conclude that CysB is not involved in the reg-

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Reference or source
AD16	VH1000 with a single-copy derivative of pAD40 (carrying the complete promoter region P _{2_{hemA}} P1 _{hemA} and all of the <i>hemA</i> gene to nt +42 of the <i>prfA1</i> gene fused to <i>lacZ</i>)	9
BW25113	<i>rrnB3 ΔlacZ4787 hsdR514 ΔaraBAD567 ΔrhaBAD568 rph-1</i>	4
GRB1776	MG1655 but <i>ttcA1 zdx2425::mini-Tn10cam</i>	G. Björk
IIG1	AD16 but <i>yciS::mini-Tn10</i>	This study
IIG2	AD16 but <i>cysB::IS10 yciS::mini-Tn10</i>	This study
IIG16	JWK1267 but <i>prfA1 zcg174::Tn10</i>	This study
IIG28	GRB1776 but <i>prfA1 zcg-174::Tn10</i>	This study
IIG29	MRA8 but <i>mmA2 zcg-174::Tn10</i>	This study
IIG44	JW0413 but <i>prfA1 zcg-174::Tn10</i>	This study
IIG46	JW0658 but <i>prfA1 zcg-174::Tn10</i>	This study
JW0413	BW25113 but <i>ΔthiI</i>	4
JW0658	BW25113 but <i>ΔmiaB</i>	4
JW1267	<i>recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻ m_K⁺) supE44 relA1/pCa24N(<i>cysB</i>)</i>	16
JW3305	BW25113 but <i>ΔtusB</i>	4
JWK1267	BW25113 but <i>ΔcysB</i>	4
MG1655	<i>ilvG rfb-50 rph-1</i>	www.genome.wisc.edu; 5
MRA7	MG1655 but <i>prfA1 zcg-174::Tn10</i>	M.R.-A. ^a
MRA8	MG1655 but <i>prfA1</i>	34
MRA475	MG1655 but <i>prfA1 ilv-3164::Tn10kan cysB::IS10</i>	M.R.-A.
MRA514	MRA8 but <i>mmE ilv-135::Tn10</i>	M.R.-A.
MRA532	MRA475 but <i>yciS::mini-Tn10</i>	M.R.-A.
MRA533	MRA475 but <i>yciS::mini-Tn10 cysB⁺</i>	This work
TH178	MG1655 <i>fadR::Tn10 mnmA2</i>	T. Hagervall
VH1000	MG1655 <i>lacIZ Δ(Mlu) rph⁺</i>	9

^a M.R.-A., M. Rydén-Aulin (strain collection).

ulation of *prfA* gene expression, and hence, suppression is not a consequence of overexpression of the mutant RF1 protein.

The suppressor phenotype in MRA475 disappears when excess cysteine is added. We wanted to test whether the sup-

pressor phenotype is a consequence of a lack of cysteine. It should be noted that all our experiments were done using LA plates, which contain an undetermined amount of cysteine. Therefore, we added cysteine to a final concentration of 100 μ M (in addition to what was already in the plate) to LA plates and tested the growth of strains MG1655, MRA7, and MRA475 at 42°C. That concentration is based on the optimal concentration determined by Neidhardt et al. (24). It was found that the suppressor phenotype in strain MRA475 was not affected (Fig. 3A and 4), while growth at 37°C was faster (data not shown). All growth experiments described in this work were done on plates, because many of the mutants grow so slowly that the risk of faster-growing revertants obscuring the experimental results is very high in liquid cultures. In a further experiment, we added increasing amounts of cysteine to the plates. When 200 μ M cysteine was added, the suppressor phenotype was weakened, and with the addition of 300 μ M cysteine, the suppressor phenotype disappeared (Fig. 3B and C, respectively). Growth at 37°C was not changed from that with the addition of 100 μ M cysteine. This result led us to speculate that it is not the lack of cysteine per se that causes the suppressor phenotype, but something dependent on cysteine. Cysteine is an important donor of sulfur to several enzymes; it also donates the sulfur needed for the formation of methionine and thus for that of *S*-adenosyl-methionine (18, 31). To test whether insufficient amounts of methionine or *S*-adenosyl-methionine cause the suppressor phenotype, we added increasing amounts of methionine with or without 100 μ M cysteine. The suppressor phenotype was not affected by increasing amounts of methionine (data not shown). Hence, we suggest that it is the lost ability to donate sulfur to some key component(s), other

FIG. 3. Test of the Ts phenotype at different cysteine concentrations. Strains MG1655 (wild type) (sector 1), MRA475 (*prfA1 cysB::IS10*) (sector 2), and MRA7 (*prfA1*) (sector 3) grown on LA plates at 42°C supplemented with either 100 μ M (A), 200 μ M (B), or 300 μ M (C) cysteine. The plates were incubated for about 36 h.

FIG. 4. Test of the Ts phenotype on LA plates at 42°C in strains with the *prfA1* mutation in combination with different mutations affecting the synthesis of thiolated nucleosides. Sectors: 1, IIG46 ($\Delta miaB$ *prfA1*); 2, IIG44 ($\Delta thiI$ *prfA1*); 3, MG1655 (wild type); 4, MRA8 (*prfA1*); 5, MRA514 (*mnmE* *prfA1*); 6, IIG16 ($\Delta cysB$); 7, IIG28 (*ttcA1* *prfA1*); 8, IIG29 (*mnmA2* *prfA1*). The plates were incubated for about 36 h.

than methionine, that causes the suppression. One such pathway is the thiolation of tRNA (Fig. 2).

Which thiolation(s) of tRNA causes the suppressor phenotype? The synthesis of all thiolated nucleosides in tRNA depends on the cysteine desulfurase enzyme (IscS), encoded by the *iscS* gene (19). Sulfur from cysteine is transferred to IscS, creating a persulfide, and the sulfur is, in turn, involved in the synthesis of thiolated nucleosides (Fig. 2). To investigate whether it is the lack of one or more tRNA thiolations that causes the suppressor phenotype, we tested whether an IscS mutant could suppress the Ts suppressor phenotype. We tried to combine a mutation in *iscS* with the *prfA1* mutation but failed. A strain with a mutation in *iscS* grows very poorly, which makes it essentially impossible to manipulate genetically.

The IscS protein donates sulfur to two distinct pathways for the thiolation of tRNA: one is a [Fe-S] protein-independent pathway and the other a [Fe-S] protein-dependent pathway (Fig. 2). To test whether the thiolation of tRNA is involved in the suppressor phenotype, strains with mutations in genes affecting each modification—*miaB* (s^2C), *thiI* (s^4U), *ttcA* (ms^2A), and *mnmA* (s^2U)—were tested for their suppressor ability. A phage lysate grown on strain MRA7 (*prfA1*) was used to transduce strains GRB1776 (*ttcA1*), JW0413 ($\Delta thiI$), and JW0658 ($\Delta miaB$), with selection for Tc^r . When the transductants were tested for growth at 42°C, we found that 40% of the transductants with *ttcA1*, $\Delta thiI$, or $\Delta miaB$ strains as the recipient were Ts, reflecting the cotransduction frequency between *prfA1* and *Tn10*. These transductants had presumably received the *prfA1* allele, which is not suppressed. To analyze the s^2 group of mnm^5s^2U , another phage lysate, grown on strain TH178 (*mnmA2*), was used to transduce strain MRA8 (*prfA1*), again with selection for Tc^r . The distance between *mnmA2* and the transposon is 1.1 min, and 0.15% of the transductants were Ts^+ , which correlates well with the distance between the two markers. This indicates that the lack of the s^2U modification causes the suppressor phenotype (Fig. 4). The presence of the

mnmA2 mutation was confirmed by transductions (data not shown).

The mnm^5s^2U modification. Transfer of sulfur from IscS to MnmA for the synthesis of mnm^5s^2U is a multistep process involving five enzymes: TusA, TusB, TusC, TusD, and TusE (Fig. 2). To confirm the result discussed above, a mutation in any of these five genes should lead to suppression of the Ts phenotype. For this reason, we transduced strain JW3305 ($\Delta tusB$) with a P1 lysate grown on strain MRA7 (*prfA1*), with selection for Tc^r . The Ts phenotype was tested, and as expected, the mutation in *tusB* could suppress the Ts phenotype caused by the mutated allele of RF1. That the *prfA1* allele is present was shown by transducing it out to a wild-type strain (data not shown).

Since our data point to the lack of the s^2 modification of the wobble U base as the suppressor, we tested whether lack of the mnm^5 modification, with s^2 maintained, would suppress the Ts phenotype. This experiment can be done because the two groups are added independently of each other (6). The MnmE enzyme is necessary for the synthesis of the mnm^5 group (10); therefore, strain MRA514 (*prfA1* *mnmE*) was tested at 42°C and found to be Ts^+ (Fig. 4). Hence, the lack of the mnm^5 group also leads to suppression of the Ts phenotype.

DISCUSSION

We have mapped and characterized a suppressor of a temperature-sensitive allele of RF1 (28, 29). We have found that the suppressor phenotype is linked to an *IS10* element inserted into the *cysB* gene. CysB is a transcription factor that activates the expression of operons coding for enzymes involved in cysteine synthesis, and it also acts as an autogenous repressor. The lack of this factor would lead to a Cys^- phenotype (22).

We have shown that lack of CysB does not lead to increased expression of the *prfA* gene; hence, suppression is not due to overexpression of the mutant RF1 protein. Instead, we have indications that suppression is caused by the lack of a component metabolized downstream of cysteine, and since the suppressed mutation affects RF1, part of the translation machinery, we asked whether suppression has to do with the synthesis of thiolated nucleosides in tRNA. There is evidence that cysteine is the only source for sulfur in the formation of the thiolated nucleosides in tRNA (2). Base modifications of tRNA contribute in several ways to the efficiency and accuracy of translation (reviewed in reference 7): by improving the efficiency of the tRNA, influencing codon choice, decreasing the codon context sensitivity, and preventing frameshifting. Analysis of *Escherichia coli* mutants defective in the synthesis of thiolated nucleosides showed that the lack of the s^2 group of the mnm^5s^2 modification of the wobble U caused the suppressor phenotype. In addition, we showed that lack of the mnm^5 group of the same modified nucleoside also suppresses the Ts phenotype. $tRNA^{Glu}$, $tRNA^{Lys}$, and $tRNA^{Gln}$ have this modification, indicating that one or more of these tRNAs are important for the suppressor phenotype.

To understand how hypomodification of some tRNAs can suppress the Ts phenotype caused by the RF1 mutant, we need to consider why the RF1 mutant is Ts. There are at least three possibilities. First, the mutated RF1 is known to terminate more slowly than the wild type (27), and it is possible that at

high temperatures there is essentially no termination at all. In such a situation, the first ribosome on the message will stall at the stop codon, and as a consequence, all ribosomes following will also be stalled, sometimes all the way back to the initiation codon (13). This could lead to a situation where many ribosomes are blocked, and hence, the cells are "starved" for ribosomes. We have tested this by comparing the amount of polysomes in a wild-type strain to the amount found in an RF1 mutant strain after incubation for 1 h at 44°C. There was no detectable difference (M. Rydén-Aulin, unpublished data), a result that may be explained by the fact that very few genes end with UAG, the codon for which RF1 is specific.

Second, the ribosome is only pausing and will eventually read through the stop codon and thus create an aberrant protein(s) that might be toxic. We do not think this is likely, since a mutation in ribosomal protein S4 that suppresses the Ts phenotype caused by the mutant RF1 at the same time increases the misreading of stop codons, in particular UAG (8). This result indicates that the Ts phenotype is not a consequence of increased misreading. Alternatively, it could be the other way around; increased misreading of UAG could suppress the Ts phenotype. However, experimental data contradict this hypothesis. The tRNA that reads UAG in *E. coli* is tRNA^{Gln} (26), and it is known that loss of the mnm⁵ modification leads to decreased efficiency of a suppressor reading stop codons (11). Therefore, it is not likely that the suppression we see is due to increased misreading of the UAG codon.

The third possibility is that the ribosome stalls at UAG stop codons (as discussed above), with the result that some proteins are not expressed. One or a few of these might be essential, and thus, the cell will die. A suppressor somehow must overcome the stalling, and as argued above, it does not do so by increased misreading. A prominent feature of mnm⁵s²U hypomodified tRNAs, though, is to induce frameshifts (1), possibly caused by poor binding of the tRNA to the ribosome (3). It has been shown that the slippage in mutants defective in the synthesis of the modified nucleoside mnm⁵s²U34 increases when one of the codons for lysine or glutamine is placed in the P site, followed by a UAG stop codon (32). Thus, if the gene that is not expressed due to stalling has a codon read by hypomodified tRNAs preceding the UAG stop codon, a frameshifting event would allow the ribosome to pass the stop codon. If a new downstream stop codon, UAA or UGA, is presented in the new frame, a functional protein might be produced and hence suppression of the Ts phenotype.

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