JOURNAL OF BACTERIOLOGY, July 1991, p. 4144–4154
0021-9193/91/134144-11$02.00/0

Vol. 173, No. 13

Salmonella typhimurium prfA Mutants Defective in Release Factor 1

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Received 31 January 1991/Accepted 19 April 1991

Mutations have been characterized that map in the *prfA* gene of *Salmonella typhimurium*. These weak amber suppressors show increased readthrough of UAG but not UAA or UGA codons. Some *hemA* mutants exhibit a similar suppressor activity due to transcriptional polarity on *prfA*. All of the suppressors mapping in *prfA* are recessive to the wild type. Two mutant *prfA* genes were cloned onto plasmids, and their DNA sequences were determined. A method was devised for transferring the sequenced mutant alleles back to their original location in *S. typhimurium* via an *Escherichia coli recD* strain that carries the entire *S. typhimurium hemA-prfA* operon as a chromosomal insertion in *trp*. This reconstruction experiment showed that the mutations sequenced are sufficient to confer the suppressor phenotype.

Translation termination and release of newly synthesized protein requires two soluble peptide chain release factors, as shown by in vitro studies of purified factors from *Escherichia coli* (reviewed in references 8 and 14). Termination at UAG (amber) codons requires release factor 1 (RF-1), whereas UGA codons respond to release factor 2 (RF-2) and UAA codons respond either to RF-1 or RF-2 (53). When a nonsense codon in the mRNA reaches the ribosomal A site, a release factor can bind to the ribosome and trigger hydrolysis of the completed peptide from ribosome-bound peptidyl tRNA. Release factor binding to ribosomes is enhanced by the cognate stop codons; in addition, an amber suppressor tRNA competes with purified RF-1 for binding to UAG-programmed ribosomes (2, 14, 21). Although such competition might indicate that release factors recognize the stop codon directly, other models suggest that they may simply facilitate base-specific interaction of 16S RNA with nonsense codons (41; reviewed in references 14 and 56). A third factor that stimulates in vitro activity of RF-1 and RF-2 has also been described (39).

The *E. coli* genes encoding RF-1 and RF-2 have been cloned (9, 61). The identity of the clones was established by using antibodies to the purified release factors. The RF-1 and RF-2 proteins share extensive homology, including 30% identical amino acids, as deduced from the DNA sequence (13); thus they probably share a common ancestor. The *prfA* gene encoding RF-1 has been mapped at 27 min and corresponds to a previously described locus of weak, recessive amber suppressors in *E. coli* (32, 48, 49, 61). Unlike suppressors with a mutant tRNA that reads nonsense as sense, suppressors with decreased activity of a release factor are expected to be recessive. The *prfB* gene encoding RF-2 maps at 62 min and is the promoter-proximal gene in an operon with the constitutively expressed lysyl tRNA synthetase (20, 28, 29). In *Salmonella typhimurium*, the recessive UGA suppressor *supK* is a mutant defective in the *prfB* gene (29, 30, 43).

Studies by Caskey's group and others (12, 14, 59, 60) have shown that the expression of *prfB* is autoregulated. Ribosomes translating *prfB* mRNA encounter an in-frame UGA codon early in the mRNA sequence, at codon 26. When RF-2 activity is limiting for termination, a programmed frameshift, which occurs at high frequency, allows translation to continue in the +1 frame, yielding authentic RF-2 protein. Thus, RF-2 regulates its own synthesis by a mechanism that is dependent on the normal termination activity of the protein.

The *hemA* gene lies directly upstream of *prfA* in both *S. typhimurium* (17) and *E. coli* (16, 33, 57). The *hemA* gene encodes the first committed enzyme in the heme biosynthetic pathway: glutamyl tRNA reductase (1). We previously demonstrated that a *hemA* insertion mutation is polar on *prfA*; that is, the two genes form an operon (17). In fact, a *hemA* insertion mutation reduces *prfA* expression to a level that cannot support growth. This observation, together with the existence of recessive temperature-sensitive lethal mutations of *prfA* in *E. coli*, indicates that RF-1 is an essential protein.

Why are the *hemA* and *prfA* genes associated in an operon? Perhaps the expression of *prfA*, like that of *prfB*, is autoregulated. On the basis of the known *prfB* regulatory mechanism, we have suggested that levels of RF-1 might be sensed by its activity at the UAG codon in which the *hemA* gene terminates (17). At present there is no direct evidence for such a model, which might invoke a variety of specific mechanisms. A closely related problem is to understand how synthesis of HemA and RF-1 proteins is coordinated by using the same mRNA, when the two proteins meet such different cellular demands. Well-characterized *prfA* mutants will be essential for testing regulatory models.

In this report, we describe the isolation and characterization of a number of *prfA* mutants of *S. typhimurium*. We also describe the design and use of a simple system based on transformation of *E. coli recD* mutants (47, 54), which allowed reconstruction of *S. typhimurium* strains carrying sequenced *prfA* mutations.

MATERIALS AND METHODS

Bacteria and phage. *S. typhimurium* and *E. coli* strains used in this study and their sources are listed in Table 1. All *S. typhimurium* strains were derived from the wild-type strain LT-2. Strains carrying transposon insertions linked to *hemA* have been described previously (19).

The *S. typhimurium* wild type does not carry the *lac* operon. The mutant *lac* operon used here to screen for *prfA* mutations is present as part of a transposon derived from phage Mu (10, 55a). This Mud phage (termed Mud F) carries a copy of the entire *lac* operon, including the *lacI* gene

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TABLE 1. Bacterial strains

Strain	Genotype or description	Source or reference
E. coli		
DPB271	*recD1903*::mini-Tet	4
MH-1	*araD139* Δ(*lac*)*X74 galU galK hsdR* (Str^r)	24
SHSP19	*hemA8 met lac* (Str^r)	51
TE2628	*recD1903*::mini-Tet *trpDC700*::*putA1301*::Kan	This study
TE2640	F$^-$ λ$^-$ IN(*rrnD-rrnE*)*1* Δ(*lac*)*X74 rpsL galK2 hemA8*	This study
TE2899	*recD1903*::mini-Tet *trpDC700*::*putPA1304*::['*prsA*-Cam-*kdsA*']	This study
TE3057	*araD139* Δ(*lac*)*X74 galU galK hsdR* (Str^r) *recD1903*::mini-Tet *hemA8 trpDC700*::*putPA1304*::['*prsA-hemA-prfA-kdsA*']	This study
TE3525	*araD139* Δ(*lac*)*X74 galU galK hsdR* (Str^r) *recD1903*::mini-Tet *hemA8 trpDC700*::*putPA1304*::['*prsA*-Δ(*hemA-prfA-dorf1*)::Kan-*kdsA*']	This study
UF261	*gyrA rpoB ara argE*(Am) Δ(*pro-lac*)/F' *pro*$^+$ *lacI-Z* (Δ14)	D. Andersson (38)
UF265	Like UF261, except UAG at *lacI* codon 189	D. Andersson (38)
UF274	Like UF261, except UAA at *lacI* codon 189	D. Andersson (38)
UF281	Like UF261, except UGA at *lacI* codon 189	D. Andersson (38)
S. typhimurium		
DB7136	*hisC527*(Am) *leuA414*(Am)	62
DB7154	*hisC527*(Am) *leuA414*(Am) *supD10*	62
DB7156	*hisC527*(Am) *leuA414*(Am) *supF30*	62
DB7303	*hisC527*(Am) *leuA414*(Am) *supC80*	62
GT2369	*divF6552 zhb-736*::Tn*10 rpsL107*	D. Hughes, G. Bjork
TE277	*zde-1858*::Tn*10d*-Tet	19
TE335	*zde-1859*::Tn*10d*-Cam	19
TE485	*his-10081*::Mud F [*lacZ455*(Am)]	This study
TE768	*araC1* DUP[(*cob-4*)* Tn*10**(*zdd-1852*)][a]	19
TE1277	*hsdL* (r_L^- m_L^+) (Fels2$^-$)? *galE542*	This study
TE1864	*leuA414*(Am) (r$^-$ m$^+$) (Fels2$^-$)/pBR322	17
TE1967	*leuA414*(Am) (r$^-$ m$^+$) (Fels2$^-$)/pRF1	17, 61
TE2470	*araC1* DUP[(*hemA702*::Kan *cob-4*)* Tn*10**(*zdd-1852*)]	17
TE2948	*putPA1304*::['*prsA*-Cam-*kdsA*']	This study
TE3059	*hemA60 his-10081*::Mud F [*lacZ455*(Am)]	This study
TE3107	*his-10081*::Mud F [*lac*$^+$] *prfA*$^+$	This study
TE3108	See Table 2	This study
TE3109	See Table 2	This study
TE3110	*his-10081*::Mud F [*lacZ455*(Am)] *prfA*$^+$	This study
TE3213	*proBA661*::Tn*10 prfA8*	This study
TE3215	*proBA661*::Tn*10 prfA10*(Ts)	This study
TE3217	*proBA661*::Tn*10 prfA*$^+$	This study
TE3604	*his-10081*::Mud F [*lacZ455*(Am)] *putA1302*::Cam *leuA414*(Am) *hsdL* (r_L^- m_L^+) (Fels2$^-$) *zde-1858*::Tn*10d*-Tet *hemA60*	This study
TE3651[b]	*his-10081*::Mud F [*lacZ455*(Am)] *prfA1*	This study
TE3652[b]	*his-10081*::Mud F [*lacZ455*(Am)] *prfA7*	This study
TE3653[b]	*his-10081*::Mud F [*lacZ455*(Am)] *prfA8*	This study
TE3654[b]	*his-10081*::Mud F [*lacZ455*(Am)] *prfA10*(Ts)	This study
TE3668	*his-10081*::Mud (Amp^r *lac*$^+$)	This study
TE3706	TR5877 pTE367	This study
TE3808	*his-10081*::Mud F [*lacZ455*(Am)] *prfA14* (NB$^-$) *zhb-736*::Tn*10 rpsL*$^+$	This study
TE3809	*his-10081*::Mud F [*lacZ455*(Am)] *prfA14* (NB$^-$) *zhb-736*::Tn*10 rpsL107*	This study
TE3810	*his-10081*::Mud F [*lacZ455*(Am)] *prfA15* (NB$^-$) *zhb-736*::Tn*10 rpsL*$^+$	This study
TE3811	*his-10081*::Mud F [*lacZ455*(Am)] *prfA15* (NB$^-$) *zhb-736*::Tn*10 rpsL107*	This study
TR1168	*lys-554 hisG200*(UGA) *supK584*	J. Roth
TR5877	*hsdL6 hsdSA29* (r_{LT}^- m_{LT}^+ r_S^- m_S^+) *metA22 metE551 ilv-452 trpC2 xyl-404 rpsL120* (Str^r) H1-b H2-e,n,x (Fels2$^-$) *nml fla-66*	B. A. D. Stocker

[a] Asterisks indicate duplication join points.
[b] Reconstructed strain. See Materials and Methods for details.

encoding the repressor, and specifies Kan^r. It is inserted into the *his* region. Its *lacZ455*(Am) derivative was constructed by homogenotization with a mutant F' plasmid from J. Roth's collection. Otherwise isogenic *lac*$^+$ and *lacZ*(Am) derivatives of *prfA* mutant *his*::Mud F [*lacZ455*(Am)] strains were constructed by transduction with donor P22 phage grown on strain TE3668. This strain carries a transposition-defective Amp^r Mud whose left end is that of Mud I-8 (26) and whose right end, including *lac*, is that of the wild-type Mud F described above. Amp^r Kan^s recombinants were selected and screened for their Lac phenotype on MacConkey-lactose plates.

Media and growth conditions. Nutrient broth (NB; Difco) and LB broth (37) were used as maximally supplemented media. NCE medium containing 0.2% glycerol was used as the minimal medium (3). Difco Bacto-Agar was added at a final concentration of 1.5% for solid media. Supplementation with 5-aminolevulinic acid was at 1 μM in minimal medium

and 150 μM in rich medium (19); amino acid supplementation was as described previously (15). Where noted, isopropyl-β-D-thiogalactoside was added at 1 mM. Antibiotics were added to final concentrations in rich or minimal medium, respectively, as follows: sodium ampicillin, 30 or 15 μg/ml; chloramphenicol, 20 or 10 μg/ml; kanamycin sulfate, 50 or 100 μg/ml; and tetracycline hydrochloride, 20 or 10 μg/ml. Except when otherwise noted, strains carrying *prfA* mutations were grown at 30°C, although they are not, in general, temperature sensitive.

Genetic techniques. The high-frequency generalized transducing bacteriophage P22 mutant HT*105/1 int-201* (52) was used for most transductions. Phage P22 lysates of *S. typhimurium* and *E. coli* strains were prepared as described previously (15, 17), except that lysates of NB$^-$ *prfA* mutants were prepared in minimal glycerol medium containing histidine. Details of P22 transduction methods were as previously described (18). Phage P1 *vir* was used for transduction between *E. coli* strains. The strain of P1 *vir* phage used was provided by Rolf Menzel and was originally from P. Bassford. P1 growth and transductions were by standard methods (55). Competent cells were prepared by standard techniques (40).

Plasmid constructions. Techniques for plasmid construction followed standard methods (5, 34). DNA fragments were purified from excised gel slices by using a GeneClean kit (Bio 101). The Kan^r fragment used for gene disruptions is derived from Tn*903* and was obtained from the plasmid pUC4K (Pharmacia). The plasmid pTE367 was constructed from pRF1 (61) by deleting DNA between the *Pvu*II site downstream of *prfA* and the *Eco*RI site of pBR322. The construct leaves an intact *prfA* gene and 137 bp extending downstream of it.

Transformation with linearized plasmid DNA. To transfer various insertion mutations and *prfA* alleles carried on plasmids to the *S. typhimurium* chromosome, we used *E. coli recD* mutant strains as recipients for transformation. These *recD* strains allow efficient recombination of linear (restriction enzyme-cleaved) DNA with homologous sequences present on the bacterial chromosome (47, 54). Plasmid DNA for these experiments was prepared by an alkaline lysis procedure (5) and sometimes was further purified by equilibrium cesium chloride density gradient centrifugation (34). For transformation experiments we used restriction enzyme-digested plasmid DNA without further purification. We selected for inheritance of a plasmid marker (either Kan^r or Hem$^+$) and, when possible, screened for loss of a chromosomal marker. When Kan^r transformants were selected, the cells were first plated on NB plates, allowed to form a light lawn, and then replica printed onto selective medium.

Nomenclature for insertions. Recombinant DNA methods make it possible to construct very complex insertions. The nomenclature used to describe constructs here is based on standard conventions. Genes are listed in the order in which they appear on the chromosome of the strain being described, but only the genes and open reading frames of interest (or known to be mutant) are shown. A double colon indicates that the material following it is a true insertion and implies that the sequence of the interrupted gene or operon resumes after the insertion. Genes that lie at the same level (i.e., that are not inserted one into the other) are separated by a hyphen. Complex insertions or extended deletions are indicated by brackets or parentheses enclosing all of the elements involved. Finally, the notation *'geneX* or *geneX'* indicates that part of *geneX* is missing on the side indicated by the prime.

This system does not specify unique strain designations. For example, all of our insertion strains have the *S. typhimurium put* region inserted between two *Bgl*II sites, one lying in the end of the *E. coli trpD* gene and the other in the beginning of the *trpC* gene. The *trp* DNA lying between these *Bgl*II sites is gone. Such a strain might be described either as Δ(*trp*)::*put* or as *trpD'-put-'trpC*.

For simplicity, we have not always specified the genus of origin of the *E. coli trp* and *S. typhimurium put* and *hemA* DNA fragments used here, since only one construction was used (see below).

Construction of strain TE3057 (*trp*::*put*::['*prsA-kdsA'*]). To facilitate other experiments not described here, we first constructed an *E. coli* strain that carries the entire *S. typhimurium put* operon inserted in the *E. coli trp* operon. The *put* DNA consists of the *Aat*II-*Pvu*II fragment of pPC6 (23) inserted between two *Bgl*II sites in the *trpD* and *trpC* genes. In addition, this *putA* gene carries a Kan^r insertion. The orientation of the insert is *trpD'-putP-putA*::Kan-*'trpC*.

Next we inserted a 7.5-kb *Hin*dIII fragment carrying the *S. typhimurium hemA* gene into the *put* operon. We were unable to construct a plasmid carrying this insertion directly, for unknown reasons. Therefore, we constructed a plasmid that carries the ends of the region we wished to transfer, flanking a drug resistance marker. The plasmid pTE360 carries the following fragments inserted into *put*: (i) a 961-bp fragment that extends from the *Hin*dIII site at codon 5 of *prsA* (7) to an *Hpa*I site in an open reading frame upstream of *hemA*; (ii) a 1.35-kb *Bam*HI fragment (filled in), carrying a Cam^r gene derived from pCJ89 (27), and (iii) an 809-bp fragment that extends from an *Hpa*I site at codon 6 of *kdsA* (16a, 63) to a *Hin*dIII site at codon 275 of *kdsA*. This construct was inserted between the *Sal*I sites in *putP* and *putA* by using *Hin*dIII linkers. The resulting *putPA*::['*prsA*-Cam-*kdsA'*] insertion was transferred to the *E. coli* chromosome by transformation of strain TE2628 (*trp*::[*putP-putA*::Kan]), selecting for inheritance of Cam^r and screening for loss of Kan^r. The resulting strain is TE2899 (*trp*::*putPA*::['*prsA*-Cam-*kdsA'*]).

We expected to finish the construction by transduction with donor phage P1 grown on an *S. typhimurium hemA*$^+$ strain, selecting for transfer of the complete *S. typhimurium hemA* region into an *E. coli* strain carrying both *trp*::*putPA*::['*prsA*-Cam^r-*kdsA'*] and a mutation in the *E. coli hemA* gene. (Recombination between homologous genes of *E. coli* and *S. typhimurium* is extremely rare and should prevent simple repair of the *E. coli hemA* gene [42].) However, the short (less than 1 kb) homology flanking the Cam^r marker apparently prevented recovery of the desired Hem$^+$ Cam^s recombinants.

To circumvent this problem, we used P22 to transduce the *trp*::*putPA*::['*prsA*-Cam-*kdsA'*] insertion into *S. typhimurium*. The resulting strain is TE2948 (*putPA*::['*prsA*-Cam-*kdsA'*]). Next, P22 phage grown on strain TE2470 was used to introduce a *hemA*::Kan insertion, selecting for Kan^r and screening for the loss of Cam^r. The *hemA*::Kan insertion cannot be inherited at the *hemA* locus because it is polar on *prfA* (17). Hence, only a small number of colonies inherited *hemA*::Kan; among these, a large fraction had lost Cam^r. After transfer of the *putPA*::['*prsA-hemA*::Kan-*kdsA'*] insertion to a *galE* host, phage P1 was grown on this strain and used to transduce the insertion back to *E. coli*. Finally, this *E. coli* strain was made *recD* and transformed with linearized pTE217 (17) to give *hemA*$^+$.

The structures of this strain (TE3057) and all of the intermediate strains in the construction have been confirmed by transduction experiments as well as by Southern hybridization (data not shown). As one illustration, strain TE3057 displays complete linkage of Trp^- and Hem^+ phenotypes. When phage P1 grown on *E. coli* TE2640 (*hemA8 trp*$^+$) was used to transduce TE3057 while selecting for Trp^+, all transductants simultaneously became Hem^-. Replacement of the *trp*::*put*::['*prsA-hemA-prfA-kdsA*'] construction with *E. coli trp*$^+$ necessarily results in the loss of the *S. typhimurium hemA* gene.

Reconstruction of *prfA* mutant strains. DNA from *prfA* mutants was cloned by using Mud-P22 hybrid phage (64), and the DNA sequence was determined as described previously (17). Both reconstructed strains (described below) carry the *Kpn*I-*Eco*RI fragment, which includes the C-terminal two-thirds of the *S. typhimurium prfA* gene (17).

The use of strain TE3525 to cross mutations from plasmids onto the chromosome, and the derivation of strain TE3525 from strain TE3057, are described in the Results. After transformation to place *prfA* mutations on the *E. coli* chromosome, they were moved into *S. typhimurium* by P22 transduction. Donor phage were grown on the *hemA*$^+$ *prfA* transformants of TE3525 and used to transduce TE3604 while selecting for Hem^+ (Table 1). Because our constructs also have homology to *put*, it was possible that some Hem^+ transductants would inherit the entire *put*::['*prsA-hemA-prfA-kdsA*'] cassette at *put* rather than replacing a smaller piece of the *hemA-prfA* region. This proved not to be the case, as shown by the retention of the *put*::Cam insertion by all Hem^+ transductants.

Duplications. Strain TE768 carries Tn*10* between its tandemly duplicated segments (at the "join point") and was constructed essentially as described previously (11, 19). The duplication starts at a site counterclockwise of *cysB* at 33.5 min on the standard map (50) and extends to the *cob-4*::Tn*10* insertion (41 min). This duplication was moved into different mutant strains by P22 transduction with selection for inheritance of Tet^r. Such Tet^r transductants carry a duplication of the same segment as that duplicated in the donor strain, but both copies of the *hemA-prfA* operon are derived from the recipient.

Mutagenesis. For localized mutagenesis with hydroxylamine (15, 25), we mutagenized a P22 transducing lysate grown on strain TE277, which carries a Tn*10d*-Tet insertion about 50% linked to the *hemA* gene, or a P22 transducing lysate grown on strain TE335, which carries a Tn*10d*-Cam insertion about 60% linked to *hemA*. These insertions map on the *prfA* (downstream) side of the *hemA-prfA* operon (17).

β-Galactosidase assays. Cultures for β-galactosidase assays were grown in minimal glycerol medium containing histidine (and other amino acids as required) at 30°C with shaking to an A_{600} of 0.5. Cells were harvested by centrifugation and resuspended in Z buffer (37) at an appropriate concentration (up to 40-fold concentrated). Cell suspensions were made permeable by treatment with sodium dodecyl sulfate and chloroform, and assays were performed as described previously (37). Assay mixtures were microcentrifuged before the A_{420} was measured. Replicate samples were incubated for various times up to 1 h, and enzyme activity was calculated from the slope of a plot of A_{420} versus time. Values for β-galactosidase activity are expressed according to Miller's formula (37). Values for β-galactosidase activity determined for the same strain on different days had standard deviations of less than 10%. The exceptions were a few cases (see Table 4) in which the standard deviation was as high as 25%.

RESULTS

Isolation of RF-1 mutants by their increased readthrough of a *lacZ* UAG codon. We screened for *S. typhimurium* mutants with decreased RF-1 activity by monitoring expression of β-galactosidase in a strain carrying an amber mutation in the *lacZ* gene. Since *S. typhimurium* is naturally Lac^-, the *lac* operon was introduced via a transposon inserted into *his* (see Materials and Methods for details). In wild-type *S. typhimurium* (or *E. coli*), the efficiency of translation termination at UAG codons is typically equal to or greater than 99.9%, although there is some variation in the readthrough at different sites (6, 38). The particular amber mutant we used, *lacZ455*, forms colonies that are just detectably blue on minimal glycerol medium containing 50 μg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. We expected that, as in *E. coli*, mutants with defects in RF-1 should act as weak amber suppressors and allow increased readthrough of the UAG codon, resulting in colonies with a darker blue color.

Localized mutagenesis was used to restrict the search to mutants that were defective in the region near the *hemA-prfA* operon. A transducing phage P22 lysate was grown on strain TE277, which carries a Tn*10d*-Tet insertion about 50% linked to *prfA*. Phage were mutagenized with hydroxylamine in vitro and used to transduce strain TE485 {*his*::Mud [*lacZ455*(Am)]} with selection for Tet^r on minimal glycerol plates containing X-gal, isopropyl-β-D-thiogalactoside, and histidine (GXIH medium) and tetracycline. Colonies that appeared darker blue were purified and tested further. The sensitivity of the screen was increased in transduction crosses, because all colonies with a *lacZ*(Am) gene, whether mutant in *prfA* or wild type, were darker blue than the parent strains on GXIH plates. This behavior is presumably due to phage P22 growth and lysis within the transductant colonies, releasing β-galactosidase into the medium.

Mutants with increased β-galactosidase activity were obtained at a frequency of 0.1 to 1% with heavily mutagenized phage preparations. A set of eight potential *prfA* mutants was obtained in this first experiment. Another *prfA* mutant (*prfA10*) was obtained by screening a collection of temperature-sensitive (Ts) mutants for the blue ($PrfA^-$) phenotype. This set of temperature-sensitive lethal mutants was isolated by using the same mutagenized phage preparation as described above.

To verify linkage to the *hemA-prfA* operon, the mutants were backcrossed, selecting the linked *hemA* marker. Transductional crosses were performed with the *prfA* mutants as donors into recipient strain TE3059 {*hemA60 his*::Mud [*lacZ455*(Am)]}, selecting for Hem^+ and screening for inheritance of the blue ($PrfA^-$) phenotype. All of the putative *prfA* mutations were tightly linked to *hemA* in this cross (observed linkages varied between 85 and 99%). Transductants that did not inherit the linked Tn*10d*-Tet were saved for further characterization (strains TE3108-1 through -8 and TE3109-2; Table 2).

Complementation with the cloned *E. coli prfA* gene. Weiss et al. cloned the *E. coli prfA* gene into pBR322 by using a screen based on the antisuppressor phenotype characteristic of RF-1 overexpression (61). In the plasmid pRF1, only about 50 bp of sequence upstream of the *prfA* gene are present. The missing DNA includes the *hemA* gene and its upstream promoter region, which are required for *prfA*

expression in its normal location in the *S. typhimurium* chromosome. The promoter responsible for expression of *E. coli prfA* in the pRF1 plasmid is not known, but enough RF-1 is made to decrease readthrough of UAG codons even in a strain whose chromosomal locus is *prfA*$^+$. Introduction of pRF1 into the *prfA*$^+$ strain *S. typhimurium* TE3110 eliminated the faint blue color shown on GXIH medium by the parent strain, whereas the control plasmid pBR322 had no effect. Introduction of pRF1 into each of the *prfA* mutant strains also gave colonies that were white on GXIH-ampicillin medium. Thus, overexpression of RF-1 from a multicopy plasmid prevents the increased UAG readthrough seen in *prfA* mutant strains. For the *prfA10*(Ts) mutant, the temperature-sensitive phenotype was also complemented by overexpression of *E. coli* RF-1.

The plasmid pRF1 contains additional DNA downstream of the *prfA* gene, including an open reading frame whose function is unknown (16a, 58a). This downstream open reading frame has been deleted in pTE367 (see Materials and Methods for details). Complementation experiments in which *prfA* mutants were tested with pTE367 gave results similar to those obtained with pRF1.

Quantitation of UAG codon readthrough. Assays of β-galactosidase activity were performed to quantitate the effect of the *prfA* mutations (Table 2). Among the nine mutants characterized initially, β-galactosidase activity was elevated 7- to 42-fold, confirming that these mutants have increased readthrough of the *lacZ* UAG codon. To eliminate possible effects at steps in β-galactosidase synthesis other than UAG codon readthrough, we compared the expression of β-galactosidase in otherwise isogenic strains carrying a particular *prfA* mutation and either a *lacZ*$^+$ or a *lacZ455*(Am) allele (Table 3). UAG codon readthrough for each strain is expressed as the ratio of β-galactosidase activity in the *lacZ* amber mutant compared with that in the *lac*$^+$ strain. This ratio indicates about 0.6% readthrough of the UAG codon in the *prfA10* mutant. Compared with a *prfA*$^+$ strain, readthrough was increased 17-fold in *prfA8* and 34-fold in *prfA10*. Thus, *prfA* mutants are weak amber suppressors. Despite their defect in RF-1 activity, these *prfA* mutants have no significant increase in generation time compared with *prfA*$^+$ strains (measured at 30°C in minimal glycerol medium plus histidine).

Other classes of mutations: *supC*, *supF*, and *hemA*. In addition to *prfA* mutations, several other classes of mutations that map in the *hemA-prfA* region are expected to increase *lacZ*(Am) readthrough. The linkage of *supC* (ochre) and *supF* (amber) suppressors to *hemA* observed in P22-mediated crosses was about 25%. This value is clearly different from that for linkage of *prfA* and *hemA* (greater than 85%). In coordinates of the *E. coli* physical map, the *hemA-prfA* operon is located at 1,278 to 1,280 kb (16a, 32), whereas *supC* and *supF* (*tyrT*) map near 1,303 kb (35).

In addition, the tRNA suppressors are substantially stronger suppressors than *prfA* mutants (see below). Since they are generated by a specific base change, they are also expected to be quite rare relative to mutations in *prfA*, which have decreased RF-1 function. Finally, *supC* and *supF* are dominant to the wild type in complementation tests, whereas all of our *prfA* mutants are recessive (see below).

The known polarity of a *hemA*::Kan insertion mutation on *prfA* expression (17) leads us to predict that *hemA* nonsense mutants might also have decreased RF-1 levels and thus allow increased readthrough of the *lacZ*(Am) mutation. Certain other *hemA* mutants might also have this property. These include frameshift mutants as well as particular nonsense mutants that were not classified as such because the HemA protein cannot function with the amino acids inserted by the nonsense suppressors tested (*supC*, *-D*, *-E*, and *-F*).

TABLE 2. Readthrough of a *lacZ*(Am) mutation in *prfA* mutant strains of *S. typhimurium*

Strain	Relevant genotype	β-Galactosidase activity[a]	Relative activity[b]
TE3108-1	*lacZ455*(Am) *prfA1*	37	31
TE3108-2	*lacZ455*(Am) *prfA2*	8.5	7
TE3108-3	*lacZ455*(Am) *prfA3*	35	29
TE3108-4	*lacZ455*(Am) *prfA4*	46	38
TE3108-5	*lacZ455*(Am) *prfA5*	50	42
TE3108-6	*lacZ455*(Am) *prfA6*	37	31
TE3108-7	*lacZ455*(Am) *prfA7*	16	13
TE3108-8	*lacZ455*(Am) *prfA8*	17	14
TE3109-2	*lacZ455*(Am) *prfA10*(Ts)	35	29
TE3110	*lacZ455*(Am) *prfA*$^+$	1.2	1
TE3107	*lacZ*$^+$ *prfA*$^+$	7,800	

[a] Cultures were grown in minimal glycerol plus histidine medium at 30°C, and β-galactosidase activity was determined as described in Materials and Methods. Activity units are those defined by Miller (37).

[b] Relative activity = (activity of *prfA* mutant)/(activity of the *prfA*$^+$ strain [TE3110]).

Eight of 12 *hemA* mutants, including 5 known amber mutants, each containing *his*::Mud [*lacZ455*(Am)], showed increases in *lacZ*(Am) readthrough on GXIH medium containing 5-aminolevulinic acid. Assays of β-galactosidase activity in several of these mutants confirmed the results seen on indicator plates. Values for UAG codon readthrough were between 0.1 and 0.5% (data not shown), in the same range as those of most of the *prfA* mutants tested above. Reversion tests were carried out on two mutants to confirm that the *hemA*(Am) mutation is responsible for the PrfA$^-$ phenotype. Some revertants selected as Hem$^+$ were very dark blue on GXIH medium and probably carried spontaneously arising nonsense suppressors, and about half were white and had restored the wild-type PrfA phenotype.

Mutants defective in *prfA* that grow poorly on rich medium (antiauxotrophs). In spite of their translation termination defect, the *prfA* mutants described above grow normally on rich medium (NB) and on minimal glycerol medium containing histidine. In a subsequent, larger search for additional alleles, we found eight *prfA* mutants that grew poorly on NB agar (NB$^-$ phenotype). The failure of these mutants to grow on NB is not due to auxotrophy for 5-aminolevulinic acid. The NB$^-$ phenotype is most clearly seen as a much lower efficiency of plating on NB agar (about 10^{-5} compared with that on GXIH). The NB$^-$ mutants also grow more slowly in minimal medium than do *prfA*$^+$ controls. For seven of the eight mutants the color on GXIH plates was darker blue than that of any other *prfA* mutant.

UAG codon readthrough levels were determined for several NB$^-$ mutants (Table 3; strains TE3832, TE3833, TE3836, and TE3837). For most, readthrough was as high as (but not substantially higher than) that observed for the most severe *prfA* mutants of the original set. But in one NB$^-$ mutant (*prfA15*), readthrough was only about fourfold higher than in the wild type (Table 3; strains TE3834 and TE3835). The existence of an NB$^-$ mutant with only modestly increased readthrough suggests that an NB$^-$ phenotype may not simply result from a very low level of RF-1 activity. It is also possible that *prfA15* is an unusual mutant.

Finding the NB$^-$ mutants suggested to us that a *hemA*::Kan insertion mutant, previously shown to be lethal in

TABLE 3. Comparison of β-galactosidase activities of *lacZ*(Am) and *lacZ*$^+$ versions of *prfA* mutant strains of *S. typhimurium*

Strain	Relevant genotype	β-Galactosidase activity[a]	Readthrough[b]	Relative readthrough[c]
TE3712	*prfA*$^+$ *lacZ*(Am)	1.4	1.9×10^{-4}	1
TE3711	*prfA*$^+$ *lacZ*$^+$	7,380		
TE3708	*prfA8 lacZ*(Am)	25	3.3×10^{-3}	17
TE3707	*prfA8 lacZ*$^+$	7,570		
TE3710	*prfA10 lacZ*(Am)	49	6.4×10^{-3}	34
TE3709	*prfA10 lacZ*$^+$	7,650		
TE3833	*prfA14* (NB$^-$) *lacZ*(Am)	68	8.5×10^{-3}	45
TE3832	*prfA14* (NB$^-$) *lacZ*$^+$	7,990		
TE3835	*prfA15* (NB$^-$) *lacZ*(Am)	6.6	8.4×10^{-4}	4
TE3834	*prfA15* (NB$^-$) *lacZ*$^+$	7,880		
TE3837	*prfA16* (NB$^-$) *lacZ*(Am)	59	7.2×10^{-3}	38
TE3836	*prfA16* (NB$^-$) *lacZ*$^+$	8,200		

[a] See footnote *a* of Table 2.
[b] Readthrough = (activity of *lacZ*(Am) mutant)/(activity of *lacZ*$^+$ strain).
[c] Relative readthrough = (readthrough of *prfA* mutant)/(readthrough of *prfA*$^+$ strain).

haploid when tested on NB medium, might be viable if tested on minimal medium. However, a repeat of the transductional inheritance test (17) showed that *hemA*::Kan mutants are not simply NB$^-$ (16a).

The NB$^-$ and PrfA$^-$ phenotypes do not segregate in transductional crosses. All NB$^-$ mutants fail to complement the PrfA$^-$ phenotype of standard *prfA* alleles (see below), and all can be complemented by plasmids expressing *E. coli* RF-1.

We used a Tn*10* 80% linked to the *rpsL* gene to construct otherwise isogenic NB$^-$ strains carrying either the *rpsL*$^+$ or the *rpsL107* (Str^r) allele (strains TE3808 through TE3811; Table 1). This Str^r allele has a restrictive effect on UAG readthrough, indicating a decreased rate of polypeptide chain elongation. The Str^r allele suppressed the NB$^-$ phenotype of *prfA14* and *prfA15*, although suppression of *prfA14* was only partial. The PrfA$^-$ phenotype was also suppressed, as assayed on GXIH indicator plates. Concurrent suppression of the NB$^-$ and PrfA$^-$ mutant phenotypes is additional evidence that they are connected.

Comparison of readthrough at different nonsense codons. It has been reported that in *E. coli*, a *prfA* mutant (originally termed *uar* [49]) shows increased readthrough of *lacZ* UAA as well as UAG codons. Indirect evidence also suggests that, in *prfA* mutants of *E. coli*, ribosomes are stalled at the *trp* attenuator polypeptide (UAA or UAG) stop codon for a longer time before release. The increased time is inferred from an increase in the transcription termination frequency, which occurs only if the stop codon is either UAA or UAG, but not UGA (44, 45).

To test effects on readthrough at each of the three nonsense codons in *S. typhimurium*, we constructed *prfA* mutant strains carrying various F′ *pro lac* plasmids as described previously (6). These plasmids were originally isolated in J. Miller's laboratory. Each plasmid carries a *lacI-lacZ* fusion (codon 356 of *lacI* is joined to codon 24 of *lacZ*). In addition, at codon 189 of *lacI* the plasmids carry either the wild-type UUA codon (leucine) or a UAG, UAA, or UGA nonsense codon. Since the identity of the amino acid inserted in the *lacI* portion of the fusion protein is not expected to affect the specific activity of the β-galactosidase produced, β-galactosidase enzyme levels ought to reflect directly the amount of readthrough at the nonsense codon.

Readthrough of the UAG codon was seen in all of the *prfA* mutants at levels comparable to those seen with *his*::Mud [*lacZ*(Am)] (Table 4). However, none of the *S. typhimurium* *prfA* mutants tested allowed detectable readthrough of a UAA or UGA codon.

Cloning and DNA sequence of *prfA* mutations. The mutations described here are partially defective alleles of an essential gene, with the exception of the conditional lethal *prfA10* mutation. Genetic mapping of these mutations is complicated by the absence of a positive selection for recombinants that have recovered the wild-type function of *prfA* (more effective termination). To verify that the mutations we isolated actually lie in the *prfA* gene, we resorted to cloning and DNA sequence analysis.

Four mutant alleles of *prfA* were cloned by using Mud-P22 hybrid phages to package DNA from the *hemA-prfA* region, as described previously (17, 64). Two of these mutant genes, derived from *prfA8* and *prfA10*, have been sequenced completely. The *prfA8* mutant gene had two changes from the wild type: Ser-4 to Phe and His-182 to Tyr. A reconstruction experiment with a DNA fragment carrying only the C-terminal two-thirds of *prfA* showed that the His-182-to-Tyr substitution is sufficient for the PrfA$^-$ phenotype (see next section and Materials and Methods). The *prfA10* mutation had changed Gly-180 to Ser. The DNA sequence changes were all GC-to-AT transitions, as expected for hydroxylamine-induced mutations.

Reconstruction of *prfA* mutant strains. To be certain that only the sequenced mutation is required for the observed *prfA* mutant phenotype, we developed a method for moving *prfA* mutations back into the *S. typhimurium* chromosome. To accomplish this we adapted the standard method of transformation with restriction enzyme-digested plasmids, using an *E. coli recD* mutant as the host. The new step in our strategy was the construction of an *E. coli* strain carrying a 7.5-kb *Hin*dIII fragment including the *S. typhimurium hemA-prfA* operon inserted into the *E. coli trp* operon. (The construct is also flanked by part of the *S. typhimurium put* operon, but this is not relevant to the present experiments.)

TABLE 4. Comparison of readthrough of *lacI* nonsense codons in *S. typhimurium prfA* mutant strains

Parent strain	Relevant chromosomal genotype	β-Galactosidase activity[a] of F′ *lacI-Z* fusion plasmid			
		lacI-Z$^+$	*lacI-Z*(UAG)	*lacI-Z*(UAA)	*lacI-Z*(UGA)
TE3213	*prfA8*	480	0.97	<0.05	1.7
TE3215	*prfA10*	550	3.2	<0.05	1.7
TE3217	*prfA*$^+$	480	<0.05	<0.05	1.5
DB7136	*sup*°	500	<0.05	<0.05	3.9
DB7303	*supC* (UAA)	720	17	16	7.0
DB7154	*supD* (UAG)	620	180	0.09	4.0
TR1168	*supK* (UGA)	500	0.3	6.1	54

[a] See footnote *a* of Table 2.

Details of the construction are given in Materials and Methods.

Strain TE3057 (Fig. 1) is a *recD* derivative of *E. coli* MH-1 (Table 1), which carries the *trp*::*put*::['*prsA-hemA-prfA-kdsA*'] insertion, including all of the *S. typhimurium hemA-prfA* operon. The insert also carries other open reading frames of unknown function. Since TE3057 has a mutation in the *E. coli* copy of *hemA*, a functional *S. typhimurium hemA* gene is required to confer the Hem$^+$ phenotype. TE3525 was constructed from TE3057 by transformation with a linearized plasmid that has a segment of *hemA* and all of *prfA* deleted and replaced by a Kan^r fragment. The resulting strain is Hem$^-$ and Kan^r (Fig. 1).

Plasmids carrying the *hemA-prfA* operon with a substituted mutant *prfA* gene were cut with *Hin*dIII, and the DNA was used to transform TE3525, selecting for Hem$^+$ and screening for Kan^s. Screening for loss of the chromosomal Kan^r marker eliminates other, undesired classes of transformants. Hem$^+$ recombinants that inherit the plasmid's *hemA* gene necessarily receive a mutant *prfA* gene as well. The reconstructed, mutant *prfA* region was then transduced into *S. typhimurium*. This gave strains that are otherwise wild type but carry a single mutation in the *prfA* gene, whose DNA sequence is known. We found that for these four cloned *prfA* mutations, including two that were sequenced, the reconstructed mutant strains were indistinguishable in phenotype from the original mutants.

Single-copy complementation tests with tandem duplications. The results described above establish that *prfA8* and *prfA10* are missense mutations in the *prfA* gene that confer a blue (PrfA$^-$) phenotype. Complementation tests with pRF1 and pTE367, described above, show that for all of the mutants described here provision of RF-1 at high levels corrects the mutant phenotype. This result does not exclude the possibility that some of the mutations giving a PrfA$^-$ phenotype actually might lie in one of the other open reading frames in the *hemA-prfA* region. Such mutants might be defective in expression of RF-1, or they might lack a protein that facilitates RF-1 action in vivo.

To test this possibility, complementation tests were carried out in which a single copy was present of each of the two alleles to be tested. The method (65) uses tandem duplications to form the necessary merodiploids, as discussed in Materials and Methods and illustrated in Fig. 2. The duplicated segment starts near and includes the *cysB* gene (33.5 min) and extends to the *cob* locus (41 min). The *hemA-prfA* operon (35 min) lies in the duplicated interval and is not linked to either end of the duplication by P22 transduction.

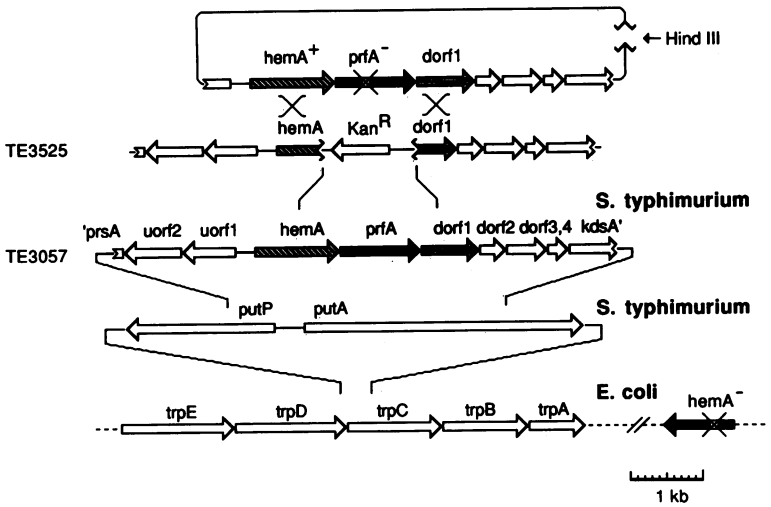

FIG. 1. Use of linear recombination to reconstruct *S. typhimurium prfA* mutants in an *E. coli* host. Strain TE3525 carries the complex insertion *trp*::*put*::['*prsA*-Δ(*hemA-prfA-dorf*)::Kan-*kdsA*'], which is described in the text. When transformed with a linearized plasmid carrying *hemA*$^+$ and a mutant *prfA* gene (top line), recombinants that have transferred the *hemA-prfA* region from the plasmid to the chromosome are selected as Hem$^+$ Kan^s. These always acquire the *prfA* allele of the donor plasmid.

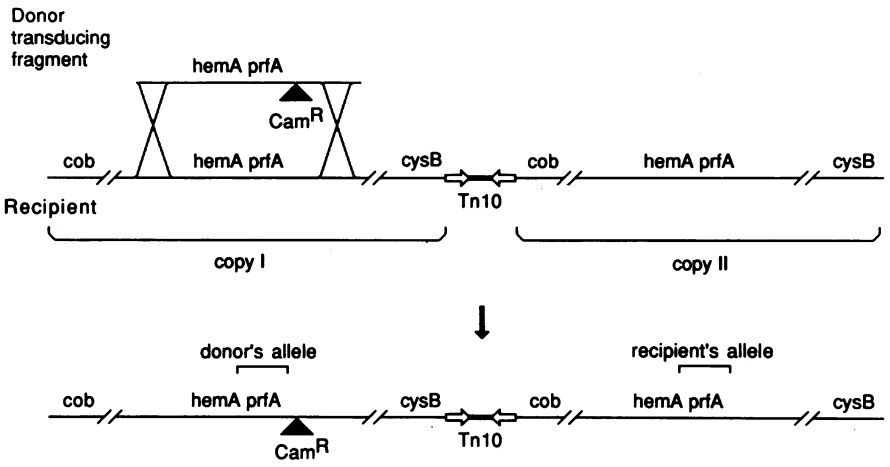

FIG. 2. Testing complementation using tandem duplications. A strain carrying a tandem duplication, including two identical copies of any given *prfA* allele, can be constructed simply by introducing a Tn*10*-held duplication join point (11). The resulting strain is shown here as the recipient. In a second, complementation cross, such a recipient strain is transduced by selecting for Cam^r with phage P22 grown on a donor *prfA* mutant strain that also carries a tightly linked Tn*10d*-Cam insertion. Most progeny (below the arrow) carry one copy of each *prfA* allele. Each recipient strain also carries the *his-10081*::Mud [*lacZ455*(Am)] insertion used to monitor UAG codon readthrough.

Homozygous diploid derivatives of *prfA* mutant strains were constructed by introducing the Tn*10* join point of this duplication with donor phage P22 grown on strain TE768 and selecting for Tet^r. To test for complementation, the resulting duplication strains were used as recipients in transduction crosses with donor phage grown on various *prfA* mutant strains carrying a tightly linked Tn*10d*-Cam insertion. In such crosses, transductants that inherit Cam^r also frequently inherit the donor *prfA* allele in one copy of the duplication (Fig. 2). Thus, the strains are heterozygous for Cam^r and *prfA*, with the general structure ($prfA_{donor}$ Tn*10d*-Cam)Tn*10* ($prfA_{recipient}$). These merodiploids permit the testing of dominance and complementation behavior.

Two important controls were carried out. First, complementation tests (within the first set of nine mutants together with the wild type) were carried out with both configurations of donor and recipient *prfA* alleles. Second, in the cases where complementation was observed ($prfA^+$/*prfA* mutant transductants), white PrfA$^+$ colonies were purified and tested for the presence of wild-type and mutant alleles. This was done by growing phage P22 on the merodiploids and transducing strain TE3059 {*hemA60 his*::Mud [*lacZ*(Am)]}, selecting for Hem$^+$ and screening for linkage of the blue (PrfA$^-$) phenotype.

Each of the *prfA* mutations was recessive to the wild type. When tested for complementation behavior, all of the mutations in the set characterized extensively above (*prfA1* through *prfA8* and *prfA10*) were found to be in the same complementation group. Since the DNA sequence and reconstruction studies show that *prfA8* and *prfA10* lie in *prfA*, all of these mutations must affect the *prfA* gene. Polar mutations in the *hemA* gene also were found to behave like *prfA* mutations in complementation tests, as expected. Most polar mutations of *hemA* can be recognized, since they are 5-aminolevulinic acid auxotrophs; however, polar mutations that are prototrophic have been observed in the *hisC* gene (58) and might occur here as well.

DISCUSSION

We have looked for mutations that map near the *hemA-prfA* operon and that allow increased synthesis of β-galactosidase in a strain carrying a *lacZ* amber mutation. The mutations isolated all affect the *prfA* gene, and all are recessive to the wild-type allele. Several temperature-sensitive lethal alleles of *prfA* were also found. Considering this together with our previous results with a *hemA*::Kan insertion, it seems likely that the mutations described here all result in a partial loss of function of the RF-1 protein.

Two of these mutations were sequenced and found to be missense alleles of *prfA*. The sequenced mutations are sufficient to confer the mutant PrfA$^-$ phenotype, as shown by reconstruction of an *S. typhimurium* strain that carries only the sequenced region in an otherwise wild-type background.

Expression of β-galactosidase in a *lacZ*(Am) $prfA^+$ strain might involve contributions from both readthrough of the UAG codon and reinitiation of translation after termination. The site of *lacZ455* is unknown, but inspection of the *lacI* gene sequence reveals no likely restart sites near codon 189 of *lacI*, where readthrough was also tested (Table 4). Readthrough might be due to misreading by a normal cellular tRNA or to reading by a suppressor tRNA mistranscribed from a wild-type gene. In any event, since RF-1 is known to play a critical role in termination, it is likely that readthrough rather than reinitiation is affected in the *prfA* mutants.

Ribosomes poised at a UAG codon may either terminate translation or read through to generate active β-galactosidase. Since RF-1 is not saturating with respect to the termination reaction in vivo (61), the rate of termination should be proportional to RF-1 activity (specific activity multiplied by concentration). We may estimate that in a $prfA^+$ strain termination at the *lacZ455* UAG codon is about 5,000-fold more frequent than readthrough (i.e., 0.02% readthrough). This estimate is probably inflated because transcriptional polarity will tend to exaggerate the effect of RF-1 action. With this simple model, we expect that a decrease in RF-1 activity to half the wild-type level should decrease the rate of termination reactions by half, so that on the average ribosomes will spend twice as long halted at the UAG codon. Doubling the number of UAG-bound ribosomes then doubles the rate of the readthrough reaction.

Individual *prfA* mutants were observed to increase expression of β-galactosidase in a *lacZ*(Am) background by 2- to 50-fold. Thus, we expect that the activity of RF-1 is decreased in these mutants by a similar factor. These substantial decreases did not appreciably affect the growth rate in minimal or NB medium at 30°C. Nevertheless, RF-1 is an essential protein.

We found an unusual class of what we call NB$^-$ *prfA* mutants, which have an antiauxotrophic phenotype. That is, they grow normally on minimal medium but do not form colonies on rich (NB) medium. Included in this class are some (but apparently not all) of those most severely defective in RF-1 activity. Mutants that grow more poorly in rich medium than in minimal medium have been observed previously, including many with changed ribosomal accuracy (36), but certain other mutants also have this phenotype (e.g., *polA* and *secB*; 27, 31). At least for mutants with altered translational machinery, it has been suggested that sensitivity to rich medium is a consequence of the heavy investment in translational components required to support high rates of protein synthesis (36).

For most translational accuracy mutants, growth is slow but is not prevented completely. In NB$^-$ *prfA* mutants the defect prevents colony formation, and suppressor mutations that allow growth are common. The ability of Str^r alleles to reduce misreading of nonsense codons is well known (22). We observed that one Str^r allele tested (*rpsL107*) reduced readthrough by NB$^-$ mutants and also suppressed the NB$^-$ phenotype.

RF-1 and RF-2 share responsibility for termination at UAA codons. In *E. coli*, a *prfA* mutation has been shown to allow increased readthrough at UAA codons in the *lacZ* gene (49). Additionally, this *prfA* mutation decreases basal expression of the *trp* operon because of an increase in transcription termination at the *trp* attenuator. This is thought to be the consequence of an increase in the amount time that ribosomes spend bound at a UAA (or UAG) codon in *prfA* mutants before release. The main evidence for the model is that the effect is specific for *trp* attenuator polypeptide genes, which terminate with UAA or UAG. Increased transcription termination is also seen in *prfB* (RF-2) mutants when a UGA codon is used (44, 45). Since wild-type *S. typhimurium* shows weak UGA suppressor activity (46), probably due to poor RF-2 function, we expected that readthrough of UAA codons in *prfA* mutants of *S. typhimurium* might be even more sensitive to changes in RF-1 activity than in *E. coli*. However, we saw no change in readthrough at the single UAA codon that was assayed.

Our studies do not show any differences between *S. typhimurium* and *E. coli* with respect to RF-1 function, with the exception of UAA readthrough observed in *E. coli* but not in *S. typhimurium*. Even this difference may not be intrinsic to the RF-1 protein but may derive instead from some other difference between the two species. We also expect that if a larger collection of *E. coli prfA* mutants were screened, NB$^-$ alleles would be found among them.

The advantages of well-characterized, sequenced, and reconstructed *prfA* mutants for genetic studies of RF-1 function are obvious. It seems particularly important to use proven missense mutants, both because the genes downstream of *prfA* are not known and because of the complex consequences for a strain in which autogenous partial suppression allows recovery of a nonsense mutation in an essential gene encoding a release factor. The *supK* UGA suppressor of *S. typhimurium* is known to be a UGA mutant of *prfB* (30), and it has also been suggested that the commonly used *prfA* (*uar-1*) mutant of *E. coli* may be an ochre mutant (49).

Finally, we should note that the single-copy complementation test, while adequate to distinguish wild type from mutant for the *prfA* alleles described here, is not sensitive enough to use with mutants having readthrough levels elevated only two- to threefold. Transductional linkage data indicate that some weaker PrfA$^-$ mutants (not described here) may lie not in the *prfA* gene, but in one of the open reading frames downstream. Further experiments will be necessary to confirm this speculation and to determine the full extent of the *hemA-prfA* operon.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM40403 from the National Institutes of Health.

We thank Qiu Yan for expert technical assistance and Tanya Kelly, who isolated some of the *prfA* mutants described here. We are also indebted to our colleagues at the University of Alabama at Birmingham for their interest and support. Bacterial strains were generously provided by Dan Andersson, Don Biek, Glenn Bjork, Kelly Hughes, Stan Maloy, John Roth, Kenneth Sanderson, Bruce Stocker, David Thaler, Bob Weiss, and Charles Yanofsky.

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