**G<sub>673</sub> could be a novel mutational hot spot for intragenic suppressors of pheS5 lesion in *Escherichia coli***

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**Abstract**

The *pheS5* Ts mutant of *Escherichia coli* defined by a G<sub>293</sub> → A<sub>293</sub> transition, which is responsible for thermosensitive Phenylalanyl-tRNA synthetase has been well studied at both biochemical and molecular level but genetic analyses pertaining to suppressors of *pheS5* were hard to come by. Here we have systematically analyzed a spectrum of Temperature-insensitive derivatives isolated from *pheS5* Ts mutant and identified two intragenic suppressors affecting the same base pair coordinate G<sub>673</sub> (*pheS19* defines G<sub>673</sub> → T<sub>673</sub>; Gly<sub>225</sub> → Cys<sub>225</sub> and *pheS28* defines G<sub>673</sub> → C<sub>673</sub>; Gly<sub>225</sub> → Arg<sub>225</sub>). In fact in the third derivative, the intragenic suppressor originally named *pheS43* (G<sub>673</sub> → C<sub>673</sub> transversion) is virtually same as *pheS28*. In the fourth case, the very *pheS5* lesion itself has got changed from A<sub>293</sub> → T<sub>293</sub> (named *pheS40*). Cloning of *pheS1*, *pheS5*, *pheS5-pheS19*, *pheS5-pheS28* alleles into pBR322 and introduction of these clones into *pheS5* mutant revealed that excess of double mutant protein is not at all good for the survival of cells at 42°C. These results clearly indicate a pivotal role for Gly<sub>225</sub> in the structural/functional integrity of alpha subunit of *E. coli* PheRS enzyme and it is proposed that G<sub>673</sub> might define a hot spot for intragenic suppressors of *pheS5*.

**Introduction**

Aminoacyl-tRNA synthetases (aaRS) play a pivotal role in translation process in all living systems. They are mainly implicated in fidelity during translation by way of attaching correct amino acid to the cognate t-RNAs (Ling et al. 2007). Although it was originally conceived that aaRS might have restricted function in aminoacylation during translation, now it is becoming increasingly evident that many synthetases possess more than one function; they are involved in cellular fidelity, t-RNA processing, RNA splicing, RNA trafficking, apoptosis, and transcription control as well as translation control (Martinis et al. 1999a,b). Multifaceted nature of aaRS has been well established in both prokaryotic and eukaryotic systems (Romby and Springer 2003; Park et al. 2005; Bori-Sanz et al. 2006; Guo et al. 2010; Smirnova et al. 2012; Guo and Schimmel 2013). Although *Escherichia coli* phenylalanyl-tRNA Synthetase comes under class II based on structural considerations, it charges the amino acid at the 2′ hydroxyl group of the...
The pheS and pheT genes that code for $\alpha$ and $\beta$ subunits of phenylalanyl-tRNA synthetase define an operon with the direction of transcription being pheS to pheT (Springer et al. 1982; Berlyn 1998). It is well known that this synthetase functions as a hetero tetramer and it is unique among the tRNA synthetases perhaps other example being glycyl-tRNA synthetase (Martinis and Schimmel 1996). Of more particular relevance to this article is the work done in our laboratory on genetics of fit loci, genes implicated in transcription control in E. coli. It has been reported that a temperature-sensitive but primarily transcription-defective mutant (fitA76) bears two lesions, one located in pheS gene and the other, named fit95, located in pheT. The pheS mutation is virtually same as that present in temperature-sensitive but translation-defective mutant pheS5 and bears a G293 → A293 transition. The other mutation fit95, by itself exhibits interesting phenotypes, but has to be present along with pheS5 in order to elicit the phenotype characteristic of fitA76 mutant. Moreover, two suppressors of fitA76 namely fitA24 and fitB have also been isolated and characterized. Even more interestingly, four rifampicin-resistant rpoB mutations have been identified which were capable of modulating phenotype of fit mutant in an allele-specific manner. All these results culminated in the notion that both $\alpha$ and $\beta$ subunits of phenylalanyl-tRNA synthetase also function as Accessory Transcription Factors (FitA and FitB) and are involved in selective gene expression in E. coli (Jabbar and Jayaraman 1976, 1978; Dass and Jayaraman 1985a,b, 1987; Munavar and Jayaraman 1987, 1993; Munavar et al. 1993; Ramalingam et al. 1999; Vidya et al. 2006; reviewed by Jayaraman 1994). Considering the emerging trends about the expanding functions of synthetases as cited above, our view that PheRS enzyme of E. coli can also function as Accessory Transcription Factor (FTT) does not look like a far-fetched view. It is this work of ours that prompted us to independently investigate the potential suppressors of temperature-sensitive primarily translation-defective pheS5 mutant (Eidlic and Neidhardt 1965; Kast et al. 1992). We hereby report the characterization of a collection of Temperature-insensitive derivatives isolated from pheS5 mutant and the analyses reveal that base pair coordinate G673 of pheS gene might define a potential hot spot for intragenic suppressors for pheS5. We propose that relevant amino acid coded by the codon affected by these mutations could be of immense importance in structural and functional integrity of $\alpha$ subunit of PheRS enzyme.

**Experimental Procedures**

**Bacterial strains, bacteriophage, and plasmids**

All E. coli strains used are derivatives of E. coli K12. P1 vir, originally from Dr. N Willetts, U.K. and maintained locally. Since most of the strains are Ts and their derivatives, depending on the nature of the experiment, the strains were grown at either 30°C or 42°C. Given in Tables S1 and S2 are the strains, plasmids used, and clones constructed for this study. Genetic Nomenclature is given according to Demerec et al. (1966).

**Media, enzymes and biochemicals**

Conventional LB medium and M9 minimal medium were used according to Miller (1972, 1992). Reagent grade materials used for preparation of various media, solutions, and buffers were purchased mainly from Hi-Media-India. Antibiotics and other fine chemicals were purchased from Sigma Company (Sigma Chemicals, St. Louis, MO). Restriction Enzymes, T4 DNA Ligase, dNTPs, and pfu polymerase were purchased from MBI-Fermentas, Genetix, Germany.

**Methods**

The molecular genetic techniques used in this study were mostly as described in Miller (1972, 1992) or with minor modifications. Most of the recombinant DNA techniques employed were according to Sambrook et al. (1989); Sambrook and Russel (2001). P1-mediated mobilization of pheS allele(s) was carried out essentially by taking the advantage of pps::Tn10 marker which is linked with pheS (60% cotransduction).

**DNA isolation, polymerase chain reaction amplification, and sequencing**

A simple and rapid method described by Chen and Kuo (1993) was followed to isolate genomic DNA from relevant E. coli strains. The following primers were used to amplify the pheS fragment pheS Forward primer 5'ATTGACTTTTATCGCCGTAGC3' and pheS Reverse primer 5' TTTGAGGAAACGCAGATCG 3', with the cyclic condition: Initial Denaturation 94°C for 5 min; Denaturation 95°C for 45 sec; Primer annealing 61°C for 45 sec; Extension 72°C for 2 min; Final extension 72°C for 5 min with pfu polymerase. The Sequencing of amplified pheS (~1.5 kb fragment) was done by Chromous Biotech, Pvt. Ltd. Bangalore, India.

**Molecular cloning of relevant pheS alleles**

Polymerase chain reaction (PCR)-based cloning strategy was followed to clone the relevant pheS alleles (Sambrook et al. 1989; Sambrook and Russel 2001). The following primers F1 and R were used to amplify and then clone the pheS region with Native and Alternate promoters;
Forward primer F1 5'CGCAAGCTTTGGTCAACGC3' and Reverse primer R 5'CGGATCCTATTATA AACTGGTGAAGTAGACCAA 3' and Reverse primer R 5'CGGATCCTATTATAAACTGGTGAAGTAGACCAA3'. The following primers F2 and R were used to amplify the pheS region only with Alternate promoter of pheST operon Forward primer F2 5'CCTTTAGTGAAGTAGACCAA 3' and Reverse primer R was designed with Hind III site and the pellet was suspended in saline and spun down, and the pellet was suspended in saline and spread on LB plate containing tetracycline (10 μg mL⁻¹) and incubated at 42°C. Six independent experiments were done to avoid getting siblings and a total of 58 Temperature-insensitive derivatives could be isolated. It is also possible that some of the temperature-insensitive derivatives of pheS5 mutant, 1ml of overnight grown saturated culture of NP37 pps::Tn10 strain was taken, spun down, and the pellet was suspended in saline and spread on LB plate containing tetracycline (10 μg mL⁻¹) and incubated at 42°C. Six independent experiments were done to avoid getting siblings and a total of 58 Temperature-insensitive derivatives were isolated. The stability of all the 58 Ts+ derivatives were checked by repeatedly growing them for several generations at 30°C and replica patching them at 30°C and 42°C. During our analyses, all the 58 Ts+ derivatives were found to be very stable.

**The suppressors of pheS5 may be tightly linked to pheS5**

Since the 58 Ts+ derivatives were isolated from six independent experiments, as stated above we would have avoided siblings. It is also possible that some of the temperature-insensitive derivatives could be True revertants in which the pheS5 (G293 → A293) lesion could have reverted back to G293 itself and thus giving rise to Ts+ phenotype. Some others could possibly be pseudorevertants and such pseudorevertants are expected to retain pheS5 lesion (G293 → A293 in pheS) and would have acquired another mutation as a suppressor. We are compelled to make a model that more than one mutation (as a suppressor) might not have emerged from our selection as they were isolated as spontaneous revertants and not by using a mutagen. Owing to the uncertainties pertaining to map position of the suppressor allele(s) we initially did Genetic analysis. For this purpose, P1 lysates were made on all the 58 Ts+ derivatives and the lysates were independently used to transduce the linked pps::Tn10 marker into a pheS strain MG1655. From all the 58 different transductional crosses, the TetR (pps::Tn10) transductants were obtained on selective plates containing LB+Tet+cit-

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**Table 1. Relative viability of relevant strains.**

| Strain/Relevant Genotype/Phenotype | Promoter(s) and pheS alleles present in relevant plasmid clones | *Relative viability CFU/mL at 42°C |
|-----------------------------------|---------------------------------------------------------------|---------------------------------|
| NP37 pheS5 Ts                     | NP and AP- pheS+                                              | 0.91 × 10⁻⁶                     |
| Ts+ derivative 1                  | NP and AP- pheS5                                              | 0.94                            |
| Ts+ derivative 2                  | 2 × 10⁻⁴                                                     |                                 |
| Ts+ derivative 3                  | 2 × 10⁻⁴                                                     |                                 |
| Ts+ derivative 19                 | 4.7 × 10⁻⁴                                                   |                                 |
| Ts+ derivative 28                 | 4.7 × 10⁻⁴                                                   |                                 |
| Ts+ derivative 37                 | 4.7 × 10⁻⁴                                                   |                                 |
| Ts+ derivative 40                 | 4.7 × 10⁻⁴                                                   |                                 |
| Ts+ derivative 43                 | 4.7 × 10⁻⁴                                                   |                                 |
| Ts+ derivative 50                 | 4.7 × 10⁻⁴                                                   |                                 |
| Ts+ derivative 55                 | 4.7 × 10⁻⁴                                                   |                                 |
| NP37 pheS5 Ts                     | Nil                                                           | <0.17 × 10⁻⁸                    |

*Relative viability refers to average values obtained in three independent experiments. NA, not applicable; NP, native promoter (Fayat et al. 1983); AP, alternate promoter (Kamalakar 2006). For further details see text and methods.
rate (see Methods). From each cross, 100 TetR transductants were taken and replica patched at 30°C and 42°C. Among the 58 transductional crosses not even from a single cross we could get a single transductant exhibiting a Ts phenotype. Evidently, in all crosses, all the checked transductants were temperature insensitive. These results strongly support the notion that all the Ts\(^+\) derivatives could be true revertants. If some were to be pseudorevertants then, the relevant suppressor in such pseudorevertant alone might not confer a Ts phenotype (See below) and it should be very tightly linked to pheS5 itself.

**Quantification of suppression of pheS5 Ts phenotype in selected 10 derivatives**

Among the 58 revertants, 10 were characterized in detail. First, in order to have a clue about the nature of suppressor (True, Pseudo) in these selected 10 derivatives, we studied the Relative level of suppression of Ts phenotype (RV). In these 10 derivatives some times, some pseudorevertants might exhibit a stable phenotype in qualitative analysis but RV values may not be 1. Therefore, all the 10 Ts\(^+\) (chosen) isolates were grown overnight in LB\(^+\)Tet and relevant dilutions were plated at 30°C and 42°C. The RV values were in the order of ~1 in all the revertants (Table 1) and this suggests that among the 10, if any or some were to be pseudorevertants, then the level of suppression of pheS5Ts mutant phenotype due to the corresponding suppressor allele should be of very good degree.

**Genetic mapping of suppressor(s) of pheS5**

In any genetic screen pertaining to isolation of revertants for a mutant, one can always expect intragenic and extragenic suppressors and also true revertants. While extragenic suppressors are predominantly associated with components of interacting system involved in a particular function/pathway (Garza et al. 1996; Rokop and Grossman 2009) intragenic suppressors are the ones which help us to know the functional domain(s)/critical amino acids in the relevant protein (Helinski and Yanofsky 1963; Davis et al. 1999; Shiomi et al. 2002). During the isolation of Ts\(^+\) derivatives of pheS5, we can expect the suppressors from pheS gene itself (intragenic) to restore the activity of PheS5 mutant protein at 42°C or the suppressors could be the activity of PheS5 mutant protein that is from its functional partner pheT (enabling the pheT mutation to form active PheRS tetrameric complex with mutated PheS5 protein) or could be in some other gene(s), the product(s) of which might function in a hitherto unreported manner so as to restore the normal function of the mutant PheS5 product. In all the above-mentioned cases, by introducing the relevant suppressor allele together with pheS5 (since it is linked with pheS5 and difficult to separate; see above) into an authentic pheS5 mutant with linked marker, one can ascertain the position of the suppressor. With this in mind, P1 lysates made on these 10 Ts\(^+\) derivatives were used to transduce the linked pps::Tn10 into a pps\(^+\) pheS5 strain and Tet\(^R\) transductants were obtained in LB\(^+\)Tet\(^+\)citrate plates (See Methods). In each cross ~100 transductants were checked for the Temperature-insensitive (Ts\(^+\)) phenotype. In almost all cases more than 70% transductants became Ts\(^+\) and grew very well at 42°C (Table 2). These results clearly indicate that the lesion responsible for the Ts\(^+\) nature of the revertants very well cotransduce with pps::Tn10 and in all likelihood the suppressor in pseudorevertants, if any, should lie between pheS5 and pps and must be tightly linked to pheS5 itself and sequence analyses reported below confirm this view.

**Sequence analyses of the pheS region from all the selected Ts\(^+\) derivatives: identification of a potential hot spot for intragenic suppressors of pheS5**

To prove the veracity of the view derived from genetic analyses, we sequenced the relevant pheS region of all the 10 Ts\(^+\) derivatives by PCR-based cyclic sequencing (See Methods). Sequence analyses were done with NCBI Blast to know the base change(s) in pheS in all the 10 Ts\(^+\) derivatives (Table 3). The sequence analyses of the 10 Ts\(^+\) derivatives and comparison of the same with pheS5/ pheS5\(^+\) sequences clearly revealed that in three Ts\(^+\) derivatives (19, 28, and 43) pheS5 lesion is intact and they have also acquired a suppressor mutation. It is very interesting to realize the fact that all the three Ts\(^+\) derivatives bear the suppressor mutation at the same base pair coordinate 673 of pheS. In fact, in two of the three cases (Ts\(^+\) derivatives 28 and 43) the base change at the position 673 is virtually same (G\(_{673} \rightarrow\) C\(_{673}\); Gly\(_{225} \rightarrow\) Arg\(_{225}\)). In the third case, the base change is G\(_{673} \rightarrow\) T\(_{673}\) (Ts\(^+\) derivative 19), which changed the amino acid from Gly\(_{225}\) to Cys\(_{225}\). On the basis of these results we are compelled to make a proposal that G\(_{673}\) might define a potential hot spot for intragenic suppressor of pheS5. In one other revertant, the base position 293 affected in pheS5 namely G\(_{293} \rightarrow\) A\(_{293}\) got changed to T\(_{293}\) (Ts\(^+\) derivative 40) leading to Asp\(_{98} \rightarrow\) Val\(_{98}\) amino acid change. We found that the rest of the other Ts\(^+\) derivatives were true revertants, in which G\(_{293} \rightarrow\) A\(_{293}\) has got reverted back to G\(_{293}\) itself (Ts\(^+\) derivatives 1, 2, 3, 37, 50, and 55) The suppressor mutation in Ts\(^+\) derivatives 19, 28, and 43 were named as pheS19, pheS28, and pheS43, respectively. In the revertant 40, we have named the lesion as pheS40. It should be noted that pheS28 and pheS43 define the same allele.
Table 2. The % Cotransduction of Ts+ Phenotype among TetR (pps::Tn10) transductants obtained in the 10 different transductional crosses in relevant strains.

| Donor/Ts+ derivative | Recipient | Selected marker/Character | Unselected phenotype | % Cotransduction |
|----------------------|-----------|---------------------------|---------------------|------------------|
| P1/Ts+ derivative 1  | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 89.1 (114/128) |
| P1/Ts+ derivative 2  | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 85.6 (154/180) |
| P1/Ts+ derivative 3  | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 80.5 (89/112) |
| P1/Ts+ derivative 19 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 86.5 (96/111) |
| P1/Ts+ derivative 28 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 80.95 (51/63) |
| P1/Ts+ derivative 37 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 87.7 (164/187) |
| P1/Ts+ derivative 40 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 82.9 (145/175) |
| P1/Ts+ derivative 43 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 84.5 (163/193) |
| P1/Ts+ derivative 50 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 89.3 (108/121) |
| P1/Ts+ derivative 55 | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 87 (147/169) |
| P1/pps::Tn10 pheS+   | NP37 Ts pheS5 pps+ | pps::Tn10 (TetR) | Ts+ | 80.87 (93/115) |

WT pheS+ linked pps::Tn10 was also used for transductional cross as control.

Table 3. Summary of sequence changes and corresponding amino acid changes in relevant Ts+derivatives.

| Strain         | Lesion present at pheS5 position | Lesions present at the position of the suppressor mutation |
|----------------|---------------------------------|----------------------------------------------------------|
|                | Base change | Aminoacid change | Base change | Aminoacid change | Inference/Source |
| pheS+ (WT)     | G293 (WT)/NR | Gly (WT)/NR | NR | NR | Ramalingam et al. (1999) and also this work |
| NP37 pheS5     | G293 → A293 | Asp98 → Gly98 | NF | NF | True revertant; this work |
| Ts+ derivative 1 | A293 → G293 | Asp98 → Gly98 | NF | NF | True revertant; this work |
| Ts+ derivative 2 | A293 → G293 | Asp98 → Gly98 | NF | NF | True revertant; this work |
| Ts+ derivative 3 | A293 → G293 | Asp98 → Gly98 | NF | NF | True revertant; this work |
| Ts+ derivative 19 | G293 → A293 | Gly98 → Asp98 | G673 → T673 | Gly225 → Cys225 | Pseudo-revertant; this work |
| Ts+ derivative 28 | G293 → A293 | Gly98 → Asp98 | G673 → C673 | Gly225 → Arg225 | Pseudo-revertant; this work |
| Ts+ derivative 37 | A293 → G293 | Asp98 → Gly98 | NF | NF | True revertant; this work |
| Ts+ derivative 40 | A293 → T293* | Asp98 → Val98 | NF | NF | Pseudo-revertant; this work |
| Ts+ derivative 43 | G293 → A293 | Gly98 → Asp98 | G673 → C673 | Gly225 → Arg225 | Pseudo-revertant; this work |
| Ts+ derivative 50 | A293 → G293 | Asp98 → Gly98 | NF | NF | True revertant; this work |
| Ts+ derivative 55 | A293 → G293 | Asp98 → Gly98 | NF | NF | True revertant; this work |

NR, not relevant; NF, not found.
*The base change is A → T but at the same position 293.

Isopropyl β-D-1-thiogalactopyranoside-induced expression of β-galactosidase in pheS5 mutant and in the suppressed derivatives

It is well known that pheS5 mutation leads to primary defect in translation at 42°C (Kast and Henneck 1991; Ramalingam et al. 1999; Sudha et al. 2001). In the pseudorevertants reported herein, one would logically expect restoration of translational defect to a considerable extent at 42°C because their RV values were in the order of ~1. One can indirectly study the same by way of measuring the expression of the candidate gene lacZ that is β-Galactosidase levels at 30°C and 42°C in pheS5 as well as in the corresponding Ts+ derivatives. Figure 1 clearly indicates that in pheS5 mutant, after shifting the culture from 30°C to 42°C within 15 min. there is about 50% reduction in the expression of β-Galactosidase at 42°C as compared to that at 30°C and this is in accordance with the expectation. In Ts+ derivatives with suppressor of pheS5, the β-Galactosidase activity at 42°C was not affected at all upon shift to 42°C as in the WT strain. These results reinforce the earlier conclusion (see above) that the suppression due to intragenic suppressor is strong in all the four cases. The Ts+ derivatives 19, 28, 43, and 40 bearing pheS5-pheS19, pheS5-pheS28, pheS5-pheS43, and pheS40 mutations were named as TPM519, TPM528, TPM543, and TPM540, respectively.
Molecular cloning of relevant pheS allele(s) with native and alternate promoter alone

Next we wanted to know the effect of pheS5-pheS19 and pheS5-pheS28 alleles in trans (when cloned in a plasmid) in a pheS5 Ts mutant. It is needless to clone the corresponding intragenic suppressor alleles alone (pheS19 and pheS28) because intragenic suppressor alleles alone usually do not complement the mutant phenotype for which they have been isolated as suppressors, in this case pheS5. In two different mutations, if one is an intragenic suppressor for the other they can suppress only in cis and will fail to complement in trans. By and large this is expected because both mutations will independently code for a nonfunctional protein. We believe this statement will hold water in our case also. However, if the pheS5 allele is cloned with the relevant suppressor alleles in a plasmid (pheS5-pheS19 and pheS5-pheS28) then seeing their effect in a pheS5 mutant will be really interesting because it would give a clue about whether the double mutant PheS protein (PheS5-PheS19 or PheS5-PheS28) will enable the formation of active PheRS complex despite the presence of single PheS5 mutant protein encoded by the pheS5 allele of the chromosome. The original promoter of pheST operon was initially identified by Fayat et al. (1983), namely Native promoter (hereinafter referred as NP) located at 368 nucleotides upstream of ATG of pheS (TTCAATA). The transcription originating from this promoter is subjected to an attenuation control and several reports are available on this aspect (Trudel et al. 1984; Springer et al.1985; Mechulam et al. 1987; Gollnick and Babitzke 2002). It is Kamalakar during his Ph.D., thesis work from this laboratory (Kamalakar 2006; B. P. Kamalakar, M. H. Munavar, and R. Jayaraman, unpubl. data), who constructed several deletions in region of pheST operon, particularly just upstream to ATG of pheS. During his analyses, he identified that the sequence “TCTAAGT” located about 45bp upstream of ATG of pheS can indeed function as a promoter and named the same as “Alternate promoter” (hereinafter referred as AP). Several lines of evidences do support the notion that the sequence proposed to function as AP might be of importance in the regulation of expression of pheST operon (Kamalakar 2006; B. P. Kamalakar, M. H. Munavar, and R. Jayaraman, unpubl. data).

Cloning of all the relevant alleles was done based on PCR cloning strategy with appropriate forward and reverse primers with HindIII and BamHI flanking restriction sites. In order to enable the cloning of pheS5-pheS19, pheS5-pheS28, pheS5, and pheS+ alleles, all the above-mentioned relevant pheS regions with indicated pheS alleles (~1.5 kb) were first amplified using the relevant primers and then cloned in pBR322 with both Native (which includes Alternate promoter as well) and with Alternate
promoter alone. The presence of pheS insert in each clone was then confirmed by double digestion with HindIII and BamHI restriction enzymes (data not shown). pheS clones bearing relevant pheS alleles with both Native and Alternate Promoters were named as follows pTPMS519 (pheS5-pheS19), pTPMS528 (pheS5-pheS28), pTPMS5 (pheS5), and pTPMS+ (Wild Type pheS+). pheS clones bearing Alternate promoter alone were named as pTPMS519A (pheS5-pheS19), pTPMS528A (pheS5-pheS28), pTPMS5A (pheS5), and pTPMS+A (Wild Type pheS+) respectively.

**Extent of complementation/suppression of Ts phenotype of pheS5 by relevant pheS clones**

In order to study the extent of complementation/suppression of pheS5 Ts phenotype by these plasmids, all the clones bearing relevant pheS regions were transformed into NP37 (pheS5) recA::cam mutant to know their effect in trans and the extent of restoration of the functional PhrS. pBR322 plasmid vector was used as control. All the relevant clone-bearing strains with appropriate controls were grown at 30°C in LB+Amp plates. Appropriate dilutions of the cultures were plated at 30°C and 42°C in LB+Amp plates. After 24 h incubation CFU/mL at 30°C and at 42°C were calculated and then the RV values were calculated (Table 1). Shown in Figure 2A and B is nature of growth of pheS5 mutant with all these clones.

As could be seen from the results, in NP37 pheS5 genetic background, the plasmid clone-bearing pheS+ allele with AP (pTPMS+A) complemented the Temperature-sensitive phenotype much better when compared with the pheS+ clone (pTPMS+). Complementation of Temperature-sensitive phenotype due to pheS5 by Plasmid clones (either cloned with NP or cloned with AP alone) bearing either pheS5-pheS19 alleles (pTPMS519 and pTPMS519A) or pheS5-pheS28 alleles (pTPMS528 and pTPMS528A) in NP37 genetic background is poor.

**Genetic evidence that both pheS19 and pheS28 mutations either do not confer Ts phenotype or could be lethal when present alone**

Initial genetic experimentation with 58 Ts+ derivatives indicated that the suppressor mutation in relevant pseudorevertants by itself may not confer a Ts phenotype (see above). But this view should be treated conjectural because, in each case/cross in which Ts+ derivatives were served as Donor and MG1655 pps+ pheS+ served as Recipient, among TetR transductants we tested only 100 colonies and did not get even a single Ts transductant (see above). However, 100 colonies might not be a sufficient number if the recombinational separation between pheS5 and each of the intragenic suppressors (pheS19 or pheS28) is less than 1%. Based on sequence analysis, the distance between pheS5 and the intragenic suppressors (pheS19, pheS28) is exactly 380 base pairs. Owing to such a tight linkage between pheS5 and the relevant intragenic suppressors pheS19 or pheS28, screening 100 colonies in the above-mentioned crosses might not have given the correct view about the phenotype of suppressor mutations that is whether pheS19/pheS28 by themselves confer any selectable phe-

Figure 2. (A) Complementation analyses of the Ts phenotype of pheS5 by relevant clones: Nature of growth of NP37 (pheS5) Ts mutant harboring relevant plasmid clone at 30°C and 42°C when patched with ~10⁵ cells, from diluted culture (Table 1). (B) Complementation analyses of the Ts phenotype of pheS5 by relevant clones: Nature of growth of NP37 (pheS5) Ts mutant harboring relevant plasmid clone at 30°C and 42°C when we patched directly from well-grown colonies/patches (Table 1).
notype or not. Perhaps one has to screen thousands of colonies in such transductional crosses so that the recombinational separation between the two mutations is possible. Such recombinational separation will allow only the coinheritance of relevant intragenic suppressor with selected marker. It is needless to study that the phenotype of pheS43 since it is same as pheS28. In case of pheS40 it cannot be Ts since the base change is at position 293 itself, and hence, it was not done. Therefore, to know the nature of phenotype due to pheS19 and pheS28 mutations, the following was done. P1 lysates were made on the strains TPM519 (pheS5-pheS19) and TPM528 (pheS5-pheS28) and both were independently used to transduce the linked pps::Tn10 marker into a pheS+ pps+ WT strain and the TetR transductants were obtained on selective plates containing LB+Tet+citr-ate. After segregation of transductants, we looked for Ts colonies among TetR transductants and the results obtained from these crosses clearly indicate that among the 1174 TetR transductants checked with TPM519 strain as donor not even a single transductant was found to be Ts (Fig. 3, Tables 4 and 5). This is true in the case of pheS28 mutation also. Among the 1097 TetR transductants checked not even a single one was found to be Ts when we used TPM528 as Donor (Table 5). Therefore, we are compelled to make a model that either the suppressor mutations (pheS19 and pheS28) by themselves do not confer a Ts phenotype or they will be lethal when present alone.

**Mapping of mutated amino acid residue on a three-dimensional structural model of E. coli PheRS (EcPheRS)**

Mutated amino acid residues have been mapped on a three-dimensional structural model of EcPheRS (Fig. 4) This is based on the crystal structure of EcPheRS, determined by Mermershtain et al. (2011) (http://www. ebi.ac.uk/thorntonsrv/databases/cgi-bin/pdbsum/GetPage.pl). EcPheRS is a tetramer consisting of two \( \alpha \) (A and C chains) and two \( \beta \) (B and D chains) subunits. Gly225 which is proposed as a mutational hot spot for intragenic suppressors of pheS5 is found to be present in H5 helic (Phe220-Phe235 [FTNLKGTLLDFLRNFF]) of \( \alpha_2 \) subunit and also in H9 helic (Phe220-Phe234 [FTNLKGTLLDFLRNFF]) of \( \alpha_2 \) subunit. We have given a figurative illustration (Fig. 5) implying the cross talk/possible interaction between the amino acids between \( \alpha_2 \) and \( \beta_2 \) subunits of EcPheRS defined by the codon affected in pheS5 lesion and the relevant amino acid change due to intragenic suppressors. But the same kind of residue interaction was not found between \( \alpha_1 \) and \( \beta_1 \) subunits of EcPheRS (Mermershtain et al. 2011).

**Discussion**

In interacting systems involved in macromolecular metabolism usually mutation(s) in one component can be compensated for by mutation(s) in other components. The mutation(s) capable of suppressing the phenotype of the original mutation are termed as suppressors. In fact, even today functional relation between two (or more) genes that is not possible to be deciphered by other means could very well emerge from suppressor analyses. While extragenic suppressors have been of immense value in identifying components of interacting systems involved in Macromolecular Metabolism, it is the intragenic suppressors that very much pave way for identifying amino acids that are crucial for the functional restoration of the mutant protein in appropriate growth conditions (Prelich 1999; Sujatha and Chatterji 2000; Hodgkin 2005; Mcclory et al. 2010). In this investigation, starting from one of the well-characterized temperature-sensitive translation-defective pheS5 mutant NP37 defined by a G293 \( \rightarrow \) A293 transition that is PheSG98D, we have reported the isolation and characterization of four pseudorevertants among 58 Ts+ derivatives. Of the four, in three Ts+ derivatives, the base position affected by the intragenic suppressor is virtually the same (base pair coordinate 673). To our surprise in two of the three cases the very base change itself is the same, G673 \( \rightarrow \) C673 transversion (pheS28 and pheS43). In one other case, the base change is defined by a G673 \( \rightarrow \) T673 transversion (pheS19). Considering the fact that these Ts+ derivatives were isolated in six independent

**Figure 3.** Schematic illustration of the P1 transductional crosses involving TPM519 (D) and MG1655 (R). Note: In this cross TPM519 (pheS5+ pheS19- pps::Tn10) was used as donor (D) and pheS5+pheS19+pps*MG1655 served as recipient (R). The first cross-over (1) is shown as a black line and the second cross-overs are shown in coloured lines. For other details, see Tables 4 and 5.
the mutations in the region encoding for the ATPase functional interaction between domains of Hsp70s, analyzed residues) to know the regions of that interact with each other. Davis et al. (1999) in an attempt to see the functional interaction between domains of Hsp70s, analyzed the mutations in the region encoding for the ATPase domain that were found to be intragenic suppressors of a lethal mutation (I485N) mapping to the peptide-binding domain of the mitochondrial Hsp70 Ssc1. Also the suppression of Ts phenotype in dnaA508 mutant was observed by intragenic suppression (Eberle et al. 1989). In an interesting study Milija et al. (1999) have reported that mutations in tRNA synthetase genes (alaS, argS, ileS and leuS) that became resistant to even GyrA inhibitor Novobiocin. Similarly, Kuo and Nakamoto (2000) showed the intragenic and extragenic suppression of the E. coli ATP synthase subunit a mutation defined by Gly-213 to Asn change and their study led to an understanding of functional interactions between residues in the proton transport site. Klein and Georgopoulos (2001), have reported the isolation and characterization of Temperature-insensitive derivatives of groEL44 Ts mutant and found that 40/46 revertants were due to intragenic suppressors. The suppressors were shown to be defective in different amino acid substitution. In fact, it is these suppressor analyses that throw more light on the enhancement of cochaperon binding with Gp31 of T4. In a different report Shiomi et al. (2002) have reported the isolation of six independent intragenic suppressors of che-moreceptor Tar (W550A) mutant of E. coli. These suppressors were subdivided into two classes. Tar carrying class I suppressors and class II suppressors. It is this study that helped them to reinforce the importance of xWxxF experiments and frequency of occurrence of suppressors’ of pheS at the same coding position GGC (base pair coordinate 673) compelled us to make a model that this base pair coordinate 673 of pheS might define a hot spot for intragenic suppressors of pheS5. Therefore, the amino acid defined by the affected codon GGC → Gly225 should be of importance in keeping up the structural/functional integrity of PheS protein. Given in Figure 6 is the alignment of amino acids of the relevant position in PheRS from different species.

Intra and Extragenic suppressors have been reported by several groups in various genes affecting various functions. As early as in 1963, pioneering studies in trp operon by Helinski and Yanofsky revealed the suppression of trpA46 mutation by intragenic suppressors in trp operon of E. coli. Miller et al. (1991) have reported about a mutant aminoacyl–tRNA synthetase that compensates for the mutation in the Major identity determinant of its tRNA. The intragenic suppressors of ftsA13 Ts mutation have been reported by Robinson et al. (1991). Tavormina et al. (1996) studied the intragenic suppressor mutations for a class of elongation-defective and termination-proficient inviable rpoB alleles (that affect highly conserved residues) to know the regions of β that interact with each other. Davis et al. (1999) in an attempt to see the functional interaction between domains of Hsp70s, analyzed the mutations in the region encoding for the ATPase protein. Given in Figure 6 is the alignment of amino acids of the relevant position in PheRS from different species.

Table 4. Predicted phenotypes and genotypes of TetR (pps::Tn10) transductants expected out of the transductional cross depicted in the Figure 3.

| Ex. No. | Donor/relevant genotype | Recipient/relevant genotype | Selected marker/character | Unselected phenotype | Frequency (TetR) | Total (TetR) |
|---------|-------------------------|----------------------------|---------------------------|---------------------|-----------------|-------------|
| 1       | P1/TPM519 (pheS5-pheS19 pps::Tn10) | MG1655 pheS5+ pps+ | pps::Tn10 (TetR) | Ts | 0/625 | 0/1174 |
| 2       | P1/TPM519 (pheS5-pheS19 pps::Tn10) | MG1655 pheS5+ pps+ | pps::Tn10 (TetR) | Ts | 0/549 |           |
| 3       | P1/TPM528 (pheS5-pheS28 pps::Tn10) | MG1655 pheS5+ pps+ | pps::Tn10 (TetR) | Ts | 0/636 | 0/1097 |
| 4       | P1/TPM528 (pheS5-pheS28 pps::Tn10) | MG1655 pheS5+ pps+ | pps::Tn10 (TetR) | Ts | 0/461 |           |

NP, native promoter; AP, alternate promoter (Kamalakar 2006).
motif and led them to suggest that the motif does not have to be located at the extreme of carboxy terminus of receptor. Rokop and Grossman (2009) have reported the isolation of intra and extragenic suppressors of Temperate-sensitive mutants in genes *dnaD* and *dnaB* involved in DNA Replication initiation in *Bacillus subtilis*. Their study has given new insights into structure–function relationship in DnaD and DnaB and interaction between both.

From this laboratory also intra and extragenic suppressors for different class of mutants affecting different functions in *E. coli* have been isolated (Vidya et al. 2006; and references cited therein). To be more specific, *fitA24* and *fitB* alleles were isolated as suppressors for the originally isolated Temperature-sensitive Transcription-defective *fitA76* mutant and suppression of transcription defect was found to be in an allele-specific manner (\(fitA76^{−} - fitA24^{+}\); \(fitA76^{−} - fitB^{+}\); \(fitA24^{−} - fitB^{+}\)), which strongly supported the

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Figure 4. Figure showing relevant amino acids and mutations in the Crystal structure of PheRS enzyme: (A) WT PheRS; (B) PheRS from NP37 showing Gly98 to Asp98 mutation; (C) PheRS from TPM519 showing Gly98 to Asp98 and Gly225 to Cys225 mutations; (D) PheRS from TPM528 showing Gly98 to Asp98 and Gly225 to Arg225 mutations; (E) PheRS from TPM540 showing Asp98 to Val98 mutation. Note: A chain-blue, c chain-magenta, B and D-yellow orange color (template of *Escherichia coli* PheRS was adapted from Mermershtain et al., 2010) and pymolwin software was used for structure prediction of PheRS in all the cases.
involvement of Fit factors in transcription (Dass and Jayaraman 1985a,b; Munavar and Jayaraman 1987, 1993; Munavar et al. 1993; Ramalingam et al. 1999). Results of Munavar et al. (2005) have paved way for the understanding of the actual mechanism behind elicitation of Alp⁺ phenotype, which emerged from the analyses of mutations that suppress lon phenotype of E. coli. Singaravelan et al. (2010) have shown that the classical amber suppressor allele of E. coli supE44 is an allele of glnX and this amber suppressor can also suppress even ochre and opal nonsense mutations to appreciable degree but only in stationary phase. Recently Shanmughapriya and Munavar (2012) have reported the suppression, specifically of MMC⁵ phenotype of lexA₃ mutant by a combination of specific rpoB₈₇-gyrA₈₇ mutations. It has been demonstrated that this unconventional DNA repair originally called ‘SIR’ (referring to SOS Independent DNA Repair) elicited by rpoB₈₇-gyrA₈₇ mutations stems from expression of uvrB in lexA₃ mutant. These rif nal alleles allow expression of uvrB but not sulA in otherwise SOS noninducible lexA₃ strain, which is believed to be unorthodox.

As could be seen from the results presented herein, the complementation of Temperature-sensitive phenotype due to pheS₅ by Plasmid clones pTPMS₅₁₉ and pTPMS₅₁₉ₐ (bearing pheS₅-pheS₁₉ alleles) and Plasmid clones pTPMS₅₂₈ and pTPMS₅₂₈ₐ (bearing pheS₅-pheS₂₈ alleles) in NP₃₇ genetic background is poor. This could be perhaps due to the fact that the active α subunit of Ec-PheRS (E. coli PheRS) encoded by the pheS₅-pheS₁₉ alleles and pheS₅-pheS₂₈ alleles, which were functional in cis could not form such active complex in trans due to the hindrance by the mutant PheS protein, encoded by the pheS₅ allele in the chromosome. Our results make us to infer that excess of double mutant protein may not be good for survival of cells at 42°C. Our suppressor analysis compels us to make a model that there could be cross talk/interaction between Gly₉₈ and Gly₂₂₅ amino acids. Moreover, on the basis of extensive complementation analyses and the results reported herein, we propose that putative Alternate Promoter of pheST operon originally identified by Kamalakar (2006), from this laboratory could indeed help in better transcription of pheS gene when compared to that of Native promoter. This is because NP₃₇ pps::Tn₁₀ recA::cam/pTPMS₅₁₉ shows better growth than NP₃₇ pps::Tn₁₀ recA::cam/pTPMS₅₁₉ₐ. We further believe and postulate that pheS alleles present in the plasmid and those in the chromosome might play a critical role in determining the extent of restoration of PheRS
enzyme activity, which truly reflects in the extent of complementation. Clones bearing just pheS5 allele alone did not complement pheS Ts phenotype regardless of presence of either Native promoter or Alternate Promoter. We do believe that to make firm conclusion about the veracity regarding the strength of the Alternate Promoter, we need to do further experiments and these are currently underway. Considering the vast majority of the literature pertaining to Informational suppression and the vital roles of such studies play in inferring the interaction between the components or domains or amino acid with in a protein, we believe that our proposal that G673 of pheS defining a hot spot for intragenic suppressors of pheS could be of immense importance.

**Conclusion**

In this investigation, by way of systematically analyzing a collection of temperature-insensitive derivatives of pheSSTs mutant, we have clearly shown, perhaps for the first time, that the intragenic suppressors of pheS define a hot spot (Base-pair coordinate 673 of pheS). This study clearly implies the importance of amino acid Gly225 in structural/functional integrity of PheS and hence PheRS enzyme. Considering the multifaceted nature of Aminoacyl-tRNA synthetases (Hausmann and Ibba 2008; Smirnova et al. 2012) and their crucial role in cellular functions (Kast et al. 1992; Safro et al. 2000; Ling et al. 2009). We do believe that our work presented herein will throw more light on EcPheRS and would help to gain further insights into the role of different domains. We also propose that this investigation will provide new directions to understand the effect of different antibacterial agents which inhibit Bacterial Translational fidelity.

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**Conflict of Interest**

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of Bacterial strains (derivatives of E.coli K12) used in this study.
Table S2. Plasmid vectors/clones, used/constructed in this study.