## BRIEF COMMUNICATION

# Suppressor Mutations Derived from the Most Severe Protein Folding Mutation Known.

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**This brief report describes the isolation and initial characterization of revertants to the most severe temperature sensitive folding mutant known. The revertants or suppressors may describe amino acid interactions that occur during the folding of the P22 tailspike polypeptide chain. Results indicate that several different types of suppressors may have been obtained.** *Key words: Tailspike protein, Hyper temperature sensitive mutant, Protein folding, P22 bacterial virus, Suppressors*

Besides being a basic question of vast importance in Biology, the problem of protein folding has taken on a more immediate relevance because in the last ten years an increasing number of maladies have been shown to have their roots in protein folding (1-4). However, to affect therapeutic intervention of these disease-related protein folding defects, a thorough understanding of how a protein folds must be achieved. Basic details such as how amino acids interact with other amino acids during protein folding will be necessary details to understand. This interaction is the focus of these preliminary results.

A simple procaryotic system (5, 6), based on the *Salmonella typhimurium* bacterial virus (phage) P22, has been developed to study protein folding. This protein folding system focuses on the tailspike protein (TSP) of the phage P22. A review on this protein folding system has recently been published (7). Mutants have been directly isolated in the protein folding process as temperature sensitive for folding mutants, *tsf* (8). These mutants do not allow for virus assembly of the intact infectious phage particle because of the defective misfolded TSP produced at high temperatures (37°C-39°C) but do assemble at lower permissive temperatures such as at 30°C. Normal wild type P22 TSP is unusual in many respects, including its very high $T_m$ of about 85°C (9, 10). Tsf mutant proteins, purified at the permissive temperature, maintained the very high $T_m$ characteristic of the wild type protein (9, 10). At the nonpermissive or restrictive temperature, the TSP accumulates as an insoluble aggregate , similar to inclusion bodies (11-14).

Using these *tsf* mutants as a starting point, some analyses have been carried out to describe amino acid interactions during protein folding by the isolation of revertants (15-17). These revertants (or suppressors) contain two mutations, the *tsf* mutation which causes a defect in the protein folding of the TSP at high temperatures, and a suppressor mutation which alleviates the damaging effect of the *tsf* mutation on the intact protein. A major result has been that there are at least two types of suppressors (mutations that alleviate defects of *tsf* mutations).

One type is the global suppressor which suppresses the defects of a large number of *tsf* mutations which are located at a variety of positions within the structure of the P22 TSP (16, 17). Two sites have been found individually to be involved in this general (global) class of suppressors, at amino acid positions 331 and 334. This global suppressor class apparently alleviates the aggregation phenomena at the nonpermissive temperature. However, at least another type of suppressor has been isolated and this report augments the very small number of mutants in this second class of suppressors (17).

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Since the 3D protein structure for the trimeric P22 TSP is known, the DNA sequence of a suppressor mutation immediately reveals if the suppressor amino acid substitution corrects the *tsf* amino acid substitution because of physical proximity (18). No *tsf /suppressor* mutation pair have been found that are in close proximity to each other.

Originally, the *tsf* mutant, to be described in this report, was isolated in studies which generated amber mutations of gene *9*, the gene for the TSP (19). The aim of this amber mutation study was to determine if the amino acids, which were inserted in the corresponding position of these amber sites in the TSP polypeptide chain by amber suppressing bacterial host cells, would yield *tsf* mutants. A P22 phage containing an amber mutation in the tailspike gene, *9amUT51*, at codon position 232 (out of 666 codons), was grown on a serine suppressor host whereupon the phage could grow at room temperature (23°C), but could not grow at 30°C (16, 19).

The *tsf* mutation, studied in this brief report, is a derivative of *9amUT51* (see Materials and Methods) and it has been termed *hyperts* or *hts* because it is temperature sensitive for folding at much lower temperatures (30°C) than all of the other *tsf* mutations (37-39°C). One of the global suppressors has been shown to alleviate its defect (16, 20 ).

This *hyperts* mutant is of interest for several reasons. This mutant is defective and is likely to produce aggregated protein at 30°C, a temperature which is permissive for all other *tsf* mutants. This mutant amino acid substitution is located on the dorsal fin which is a loop (containing amino acids 197-259 [out of total of 666]). This loop contains a disproportionally large number of positions which have yielded *tsf* mutants (10/37, see ref #7). It is of considerable interest to determine what makes the *hyperts* site extremely sensitive to temperature perturbations than the other *tsf* sites in the loop. Since these suppressors correct the most lethal temperature sensitive mutation known, one or more of the isolated suppressor mutations may create an extremely stable protein, perhaps even more stable than the wild type TSP ($T_m$ of about 85°C). For these reasons a study was initiated to isolate and characterized suppressors of the *hyperts* mutant. Preliminary findings are presented.

## Materials and Methods

**Bacterial strains and bacterial viruses (phages).** *Salmonella typhimurium LT2* derivative BV4010 (same as TP104 or MS1017) contains the following genotype: *leuAam414 supO (su-)* which is our suppressor negative wild type strain while *Salmonella typhimurium LT2* BV4012 (same as TP275 or MS1363) has the following genotype *leuAam414 supE (gln)*, containing the gln suppressor which allows many amber mutants to function. These strains were originally the generous gifts of Drs. Jonathan King of the Massachusetts Institute of Technology and Tony Poteete from the University of Massachusetts (MIT).

*Salmonella typhimurium* phage P22 *c1-7* served as our wild type phage. The tailspike gene of the P22 phage is gene *9*. The *hts* phage mutant used in these studies is a revertant, *tsR(am51)-e* , of the P22 phage containing the allele, *9amUT51* (20). This revertant eliminates the amber phenotype and so is presumed to have mutated the amber codon itself at position 232 of this mutant. It is also likely that this mutation at the amber codon has been replaced with a serine codon since this is the only amino acid which has been shown to yield this *hts* phenotype (15). Since the mutation of *9amUT51* has been determined to be the amber codon TAG (16), a single nucleotide change that would result in a serine codon would be the transversion, A>C, yielding the serine codon TCG. Studies are in progress to verify this supposition (K. A. Liberatore and R. J. Villafañe, unpublished data). Revertants, isolated from *hts*, have been designated, *htsR1*, *htsR2*, *htsR3*, *htsR4* and *htsR5*. The initial *hts* phage strain was a generous gift of Dr. Jonathan King (MIT).

**Preparation of a phage stock.** An overnight bacterial culture was sterilely diluted 1:100 into a flask containing 30mL of LB broth. To this flask was added one plug of a fresh phage plaque. The flask containing the diluted bacterial suspension and the phage plug was then incubated by shaking in a New Brunswick Scientific G-76 shaker bath at room temperature for *hts* phage mutant or at 30°C for the *hts* revertants, *htsR* strains. The *hts* phage mutant required at least 6 hours for visible lysis while the revertants required between 2-4 hours. One milliliter of chloroform was added to each flask after visible lysis or after the time that visible lysis normally occurs. The flasks were shaken for an additional ten minutes and the lysed cells were decanted into sterile 50mL plastic Oak Ridge tubes with care taken to avoid decanting the chloroform. The lysed cells were centrifuged in a SS34 rotor for ten minutes at 5,000 rpm at 4°C to pellet cellular debris. The supernatants were decanted into fresh sterile 50mL plastic Oak Ridge tubes and centrifuged at 15,000 rpm for ninety minutes at 4°C in the same rotor which results in pelleting of the phage particles. The resultant pellets were resuspended in 3mL of T2 Buffer (21).

The concentration of the phage stocks, the titer, was determined by the usual plate overlay method using

dilution of the phage stocks. The titer for P22 *hts* phage was determined on BV4012 by incubating the plates at 23°C ( room temperature) and at 30°C for all other phages. Phage titers were usually in the range of $10^9$ to $10^{10}$ plaque forming units per milliliter.

**Isolation of *hts* revertants.** Approximately one million phage particles of P22 *hts* were added to one drop (~50mL) of an overnight culture of BV 4010 bacterial cells and this mixture was added to top agar and plated on petri plates containing LB-agar (21). The plates were incubated for 16 hours at 30°C, a temperature restrictive for these phage. The ten resultant plaques were picked individually and purified by several cycles of plating, and plaque picking. Five of these plaques were made into phage stocks and analyzed physiologically.

**Temperature characterization of the hts and hts revertant phage strains.** The concentration of each phage strain was determined at various temperatures: 23°C, 30°C, 37°C, and 39°C. Only 23°C was permissive for the *hts* phage strain.

**Characterization of the heat stability of the intact viral structural proteins *in vitro*.** The survival of the phage particles is measured after incubation of wild type and mutant phage stocks at different temperatures: 30°C and 60°C. Samples were removed after incubation for 1hr, 2hrs and 3hrs. The 30°C incubation temperature is the control temperature. At this temperature, phage is considered reasonably stable.

## Results and Conclusions

**Isolation of revertants of P22 *hts*.** As with the other *tsf* mutants, the defect in the P22 *hts* is known to be in the folding pathway of the P22 TSP (20). Therefore, intragenic revertants of this defect would be expected to affect a repair along the folding pathway as well. DNA sequencing of the tailspike genes of these revertants should yield two mutations. One mutation would be the initial *hts* mutation while the other mutation should yield the additional suppressor mutation. P22 *hts* phage was plated one at the restrictive temperature of 30°C and incubated overnight at this temperature (see Materials and Methods for details). The more than 99.9% of the phage were unable to form plaques. Ten plaques were picked and purified. Half of these were studied in more detail as described in this brief communication (Table 1) .

**Preliminary physiological growth characterization of the P22 *hts* phage and revertants.** The growth of these mutant phages might well be affected by temperature of growth such as 23°C, 30°C, 37°C, and 39°C. Such an effect may allow a distinction between the various suppressors into different suppressor classes. Since

**Table 1.** Mutants Studied in this Report

| Hts mutants | Plaque morphology |
|---|---|
| P22 WT | Large, Turbid |
| P22 *hts* | Medium, Clear |
| P22 *hts R1* | Large, Clear |
| P22 *hts R2* | Small, Clear |
| P22 *hts R3* | Medium, Clear |
| P22 *hts R4* | Small, Clear |
| P22 *hts R5* | Very Large, Clear |

presumably the only difference between these phage mutants is the suppressor mutation, any differences in growth are a reflection the ability of the particular suppressor mutation to suppress the *hts* mutation.

The results of this study can be seen in Figure 1. Here it can be noted that for wild type phage there is no effect of temperature on phage growth. This characteristic of wild type phage growth is also shared by P22 *htsR1*, P22

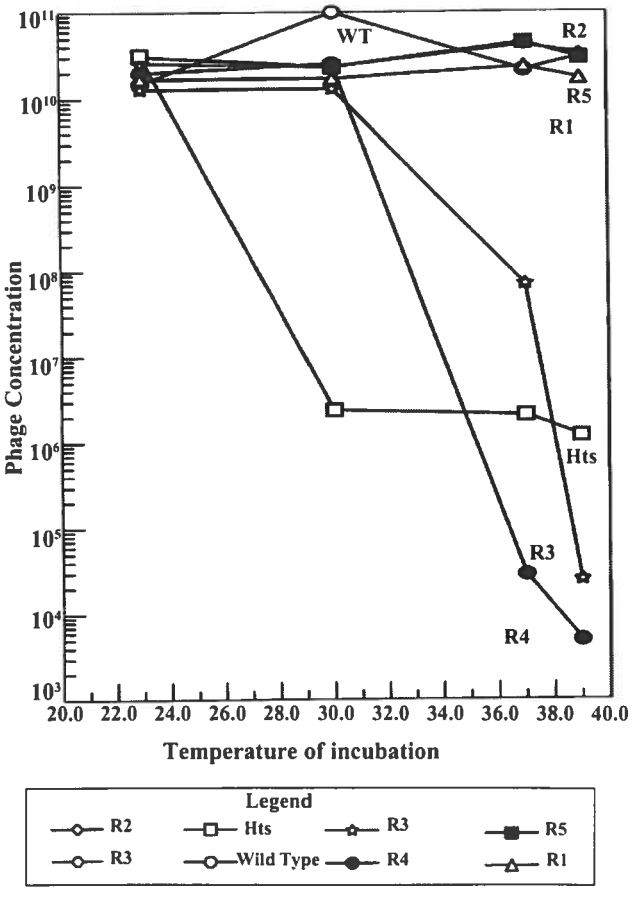


**Figure 1.** Plating behavior of P22 hts and suppressor derivatives at different temperatures (23°C, 30°C, 37°C, and 39°C).

*htsR2* and P22 *htsR5*. As expected, the growth characteristics of P22 *hts* showed that it survives at least four orders of magnitude less well at 30°C then does wild type phage, for which this temperature is optimal. The *hts* phage survives at least four orders of magnitude less well at 30°C then it does at 23°C. The P22 *hts* mutant maintained its four orders of magnitude lower growth at 37°C and 39°C.

All revertants isolated, grew at least four orders of magnitude better than P22 *hts* did at 30°C. The P22 *htsR3* and P22 *htsR4* phage mutants produced interesting phenotypes at 37°C and 39°C. They could be physiologically separated from each other at 37°C. At this latter temperature, P22 *htsR3* grew two order of magnitude better than the P22 *hts* mutant while P22 *htsR4* grew two orders of magnitude worst than the P22 *hts* mutant. At 39°C both phage suppressor mutants grew similar to each other and worst than P22 *hts*.

This study allows the preliminary characterization of at least three different classes of suppressor mutations. One class of mutants is not affected by temperature while the two other classes are distinguished by growth characteristics at 37°C. These differences are likely to be borne out at the level of DNA sequence.

**Preliminary characterization of the stability of the phage particles from the P22 hts and revertants mutants.** Because of the severe defect in the *in vivo* maturation of the P22 *hts* at relatively low temperatures, it was of interest to determine if this amino acid substitution affected the stability of the TSP structure when it was part of the mature phage particle. Phage particles were incubated for three hours at different temperatures (30°C and 60°C) and an aliquot was taken every hour to check for phage survival (an indicator of TSP function). The concentration of the treated phage was determined at 30°C. The results indicate that there was no statistically significant change in the survival of the phages incubated at these temperatures. There was less than an order of magnitude change in survival. Our preliminary conclusion is that the TSP from wild type phage or from hts and its derivatives are stabile to high temperature even though *in vivo* they can be very sensitive to slight temperature fluctuations.

## Resumen

Este breve articulo describe el aislamiento y caracterización inicial de revertidores de los mutantes de plegamiento mas severos y sensitivos a temperatura. Los revertidores o supresores pueden indicar interacciones que ocurren durante el plegamiento de la cadena polipeptídica de la cola del bacteriofago P22. Los resultados indican que varios tipos deferentes de supresores se pudieron haber obtenido.

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