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A Temperature Sensitive Nonsense Mutation Affecting the Synthesis of a Major Protein of *Escherichia coli* K12

Stephen Cooper and Therese Ruettinger

Department of Microbiology, University of Michigan School of Medicine
Ann Arbor, Michigan

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Summary. A temperature sensitive nonsense (TSN) mutant of *E. coli* K12 has been isolated in which a major bacterial protein is not synthesized at 42° C. This protein is found in the parental strain at 42° C and in cells rendered temperature resistant due to the insertion of a number of different nonsense suppressors or the normal allele of the mutant locus.

Introduction

Beckman and Cooper (1973) have described the isolation of a new class of conditional lethal mutations which they have called temperature sensitive nonsense (TSN) mutations. TSN mutations are nonsense mutations in essential bacterial functions which render a cell temperature sensitive in the presence of a temperature sensitive suppressor. They were isolated as temperature sensitive cells which were rendered temperature resistant by the introduction, by transduction or mutation, of temperature insensitive suppressors.

The main theoretical difference between TSN mutations and the classical temperature sensitive missense (TSM) mutations is that with TSM mutations the affected protein is presumably synthesized although unable to function at the nonpermissive temperature, but with TSN mutations the affected protein would not be synthesized at the nonpermissive temperature. This is because at the nonpermissive temperature the temperature sensitive suppressor is inactivated and protein synthesis does not proceed past the nonsense mutation thus leading to the production of only a fragment of the affected protein (Whitfield, 1972).

We wish to describe the isolation of a temperature sensitive cell which contains a TSN mutation as shown by numerous criteria, and which does not synthesize a major protein at the nonpermissive temperature. Temperature resistant cells obtained from the mutant by insertion of the nonmutant allele or known nonsense suppressors synthesize this protein at the elevated temperature.

This mutant provides biochemical evidence to support the previous purely genetic description of TSN mutations. This mutant also indicates the potential use of TSN mutations in identifying proteins associated with essential bacterial functions or structures. Nagata and Horiuchi (1974) have also reported the isolation of temperature sensitive cells which are due to TSN mutations.

Materials and Methods

Bacterial Strains

The strains of *Escherichia coli* K12 employed in this study are listed in Table 1. The gene designations are those of Taylor and Trotter (1972) and Fig. 1 shows the genetic map of

Table 1. Description of strains

Bacteria	F character	Genotype	Source
PNG 46	Hfr	*pho*$_{amber}$, *sup4*^{ts}	Gallucci (see Gallucci, Pachetti and Zangrossi, 1972)
PNG468	Hfr	*pho*$_{amber}$, *sup4*^{ts}, *str*	spontaneous streptomycin resistance
LS628	F$^-$	*lac*$_{amber}$, *ilv*, *trp*$_{amber}$, *mal*$_{amber}$, *str*	B. Low
H12	Hfr	*pho*$_{amber}$	A. Garen
SC121	F$^-$	*lac*$_{amber}$, *trp*$_{amber}$, *pho*$_{amber}$, *mal*$_{amber}$, *str*	Mating H12 and LS628
SC122	F$^-$	*lac*$_{amber}$, *trp*$_{amber}$, *pho*$_{amber}$, *sup4*^{ts}, *str*, *mal*$_{amber}$	transduction of SC121 with P1 grown on PNG468
KL14	Hfr	*thi*, *rel*	B. Low
BW113	Hfr	*met*, *thi*	B. Low
4248	F′	*arg*, *met*, *his*, *leu*, *rec*A, *mtl*, *xyl*, *mal*, *gal*, *lac*, *str*, *ton*, *tsx*, λ^r, *sup*E/F′141	Coli Genetic Stock Center (C. G. S. C.)
4289	F′	*arg*, *met*, *his*, *leu*, *rec*A, *mtl*, *xyl*, *mal*, *gal*, *lac*, *str*, *ton*, *tsx*, λ^r, *sup*E/F′140	C. G. S. C.
KH6	F′	*trp*E$_{amber}$, *tyr*$_{amber}$, *arg*, *met*, *rec*A, *his*/F′*his*$^+$ *sup*D$^+$	K. Horiuchi (see Nagata and Horiuchi, 1973)
KH17	F′	*trp*D$_{amber}$, *tyr*$_{amber}$, *arg*H, *met*B, *rec*A, *his*/F′*his*$^+$	K. Horiuchi (see Nagata and Horiuchi, 1973)
5349	F′	*trp*, *his*, *arg*, *rec*A, *lac*Z53, *rel*-1, *nal*A/F′196 *sup*D$^+$	C. G. S. C.
5351	F′	*trp*, *his*, *arg*, *rec*A, *lac*, *rel*, *nal*A/F′141 *sup*U$^+$	C. G. S. C.
4258	F′	*arg*, *met*, *his*, *leu*, *rec*A, *mtl*, *xyl*, *mal*, *gal*, *lac*, *str*, *ton*, *tsx*, λ^r, *sup*E/F′111 *sup*N$^-$	C. G. S. C.
4302	F′	*thi*, *his*, *aro*, *pro*, *rec*A, *xyl*, *mal*, *tsx*/F′148 *sup*D$^-$	C. G. S. C.

E. coli K12 as adapted from Taylor and Trotter (1972) with those markers and strain characteristics pertinent to these experiments. *Sup*$^+$ indicates the presence of suppressor activity and *sup*$^-$ indicates the absence of suppressor activity. In addition to the strains listed above, this work was facilitated by the use of a set of Hfr strains and a set of F′ strains which were used in the mapping studies of TSN mutations. These strains were prepared by B. Low and sent to us by B. Bachman of the Coli Genetic Stock Center. 5351 and 5349 were originally prepared by M. Oeschger. *Sup*U is described by Soll and Berg (1969).

Bacteriophage Strains

ϕ80p*sup*III$^+$ was obtained from Dr. J. D. Smith (Russell, Abelson, Landy, Gefter, Brenner and Smith, 1970). ϕ80p*sup*III$^-$ is a nitrosoguanidine (NG) induced derivative of ϕ80p*sup*III$^+$ selected as a white plaque on indoxyl-galactoside plates (Russell, *et al.*, 1970). It can spon-

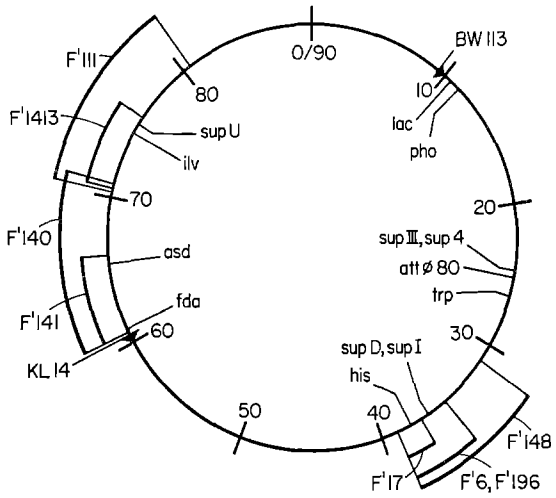

Fig. 1. Genetic map of *Escherichia coli* K12 with locations of different genetic markers used in this study. The arrows on the circle indicate insertion points and direction of transfer of Hfr strains. The arcs indicate the different F-prime episomes used for identification of TSN mutations and for mapping

taneously revert to *sup*III$^+$ and therefore is presumably altered only in the genetic material determining the suppressor function.

Media

LB broth and plates were used for all genetic manipulations (Beckman and Cooper, 1973). Labeling of cells was performed in a minimal-glucose medium with all amino acids present except valine and leucine (Cooper and Ruettinger, 1975).

Genetic Procedures

Interrupted mating experiments were performed essentially as described by Miller (1972). Introduction of F′ factors was achieved by growing the donor and recipient strains in broth at 30° C and then streaking out the liquid culture on a selective plate at 42° C. Nalidixic acid or streptomycin was used to select against donor bacteria.

Analysis of Bacterial Proteins by Polyacrylamide Gel Electrophoresis

The methods for labeling the cells, solubilization of proteins, electrophoresis on polyacrylamide gels, drying the gels, and autoradiography have been described by Cooper and Ruettinger (1975). The methods used are those developed by Laemmli (1970), Fairbanks, Levinthal and Reeder (1965), Weber and Osborn (1969), and G.F. Ames (1974). Briefly, cells grown at 30° C in minimal medium supplemented with all amino acids except valine and leucine were labeled with either ^{14}C-leucine or ^{14}C-valine 15 minutes after transfer to 42° C. The labeling period was generally 15 minutes. The cells were collected by centrifugation, solubilized with SDS-mercaptoethanol, and the proteins separated by electrophoresis in a discontinuous polyacrylamide slab gel. After electrophoresis the gel was dried onto a filter paper under vacuum and autoradiographed with Kodak No-Screen X-ray film.

Isolation of Cell Envelopes

The method of Chai and Foulds (1974) was used, which involves fragmentation of the cells in a French press and centrifugation of the lysate at 18000 RPM for 1 hour to pellet the cell envelope.

Table 2. Comparison of TSN and TSM mutations

	TSN		TSM	
	Growth, 42° C	Sup$^+$, 42° C[a]	Growth, 42° C	Sup$^+$, 42° C[a]
Reversion	+	+/−[b]	+	−
Transduction (P1)				
sup$^+$ donor	+	+/−	+	−
sup$^-$ donor	+	−	+	−
Lysogenization				
ϕ80p*sup*III$^+$	+	+	−	n.d.
ϕ80p*sup*III$^-$	−	n.d.[c]	−	n.d.
F-duction				
F′(*sup*$^+$)	+	+	−	n.d.
F′(*sup*$^-$)	−	n.d.	−	n.d.

[a] Denotes whether or not any suppressor activity exists in cells growing at 42° C. This activity can be detected by the observation of a *trp*$^+$ or *lac*$^+$ phenotype in the temperature resistant cells derived from mutant cells which contained suppressor sensitive mutations in their *trp* or *lac* genes.

[b] +/− indicates that two types of cells are observed among the temperature resistant population.

[c] n.d. indicates not determinable.

Identification of TSN Mutations

We find that approximately 1–3% of a collection of temperature sensitive cells are of the TSN class. Therefore the main problem in the isolation of TSN mutants is to distinguish them from the majority of temperature sensitive cells of the TSM class. Table 2 compares four tests that can be used to identify TSN mutations.

Beckman and Cooper (1973) studied revertants and transductants of temperature sensitive cells to classify mutants as TSN or TSM. Note that in both cases the classification of the temperature resistant revertant or transductant population, rather than the finding of transductants or revertants themselves, leads to the identification of a TSN mutation. Only with a TSN mutant will a significant proportion of the revertants or transductants (using a *sup*$^+$ donor) be found to be *sup*$^+$ at 42° C. This is because TSN mutants can become temperature resistant *either* by changing a mutation at the original mutant site *or* by the insertion of a temperature insensitive and presumably dominant suppressor by transduction or reversion. In contrast, TSM mutants become temperature resistant *only* by altering the original mutant site, with no change in the suppressor characteristics of the cells.

We have developed two additional tests for the identification of TSN mutations which are simpler and more suited to large scale screening of temperature sensitive cells. The main difference between these tests and the study of transductants and revertants is that instead of studying the phenotype of temperature resistant cells, all that has to be observed is whether or not there are temperature resistant cells (Table 2). The lysogenization test and the F-duction test involve inserting a temperature insensitive suppressor into a mutant cell and seeing whether temperature resistant cells are produced. Appropriate controls are performed to eliminate temperature resistance due to the insertion of the normal allele of the temperature sensitive mutation. The tests are performed as follows:

(a) Lysogenization Test. An LB agar plate is spread with 0.1 ml of an overgrown culture of the temperature sensitive mutant, and a drop each of ϕ80p*sup*III$^+$ and ϕ80p*sup*III$^-$ (at a concentration of 10^9 pfu/ml) are placed on the plate. After incubation of the plate at 42° C overnight, the presence of a large mass of growth with the ϕ80p*sup*III$^+$ phage and not with the ϕ80p*sup*III$^-$ phage indicates the presence of a TSN mutation.

(b) F-duction Test. A temperature sensitive mutant is grown in broth with a donor cell which contains an F′ factor which carries a suppressor. Control tubes are prepared with donor bacteria which contain F's which cover the same genetic region but which do not carry a suppressor allele. After overnight growth at 30°C the cells are struck out on LB agar and incubated at 42°C. The finding of temperature resistant F-ductants with the suppressor containing F's and not with the control F's indicates the TSN character of a mutant. This test has been used primarily for confirmation of TSN mutants found either by the reversion or lysogenization tests, but it can be used for the primary screening.

(c) Reversion Test. A population of temperature resistant revertants (obtained by plating 10^7–10^8 cells on LB agar and incubating at 42°C) are examined for their suppressor activity by either or both of two different tests. (i) The revertant cells are replicated onto minimal plates which are incubated at 42°C to determine whether cells are phenotypically *trp*$^+$ or *trp*$^-$. (ii) The revertants are produced on plates containing isopropylthiogalactoside (IPTG) and the colonies appearing at 42°C are stained for β-galactosidase using naphthylgalactoside and Fast Blue RR as described by Miller (1972). The finding of two different types of colonies in either or both tests is taken as indicating the presence of a TSN mutation.

Isolation of tsn-K165

Strain SC122 was mutagenized with 1.0 mg/ml of nitrosoguanidine (NG) (Russell, Abelson, Landy, Gefter, Brenner, and Smith, 1970), resuspended in fresh broth and allowed to grow at room temperature overnight. The mutagenized cells were plated out for colony formation at 30°C and replicated to 42°C. Temperature sensitive cells were picked and purified and classified as TSM or TSN. Temperature sensitive mutant strain *tsn*-K165 was one of five TSN mutants isolated from that mutagenesis as revealed by the lysogenization test. The TSN character of the mutant was confirmed by finding that the temperature sensitivity of the mutant was eliminated by F-duction with any of three different F's containing two different suppressor alleles. (A nalidixic acid resistant derivative of *tsn*-K165 was prepared for the F-duction tests as some of the F′ strains used were streptomycin resistant).

Results

Identification of tsn-K165 as a TSN Mutant

tsn-K165 is a temperature sensitive cell which can become temperature resistant after insertion of a suppressor (Table 3). Lysogenization with φ80 p*sup*III$^+$ phage produces temperature resistant cells, whereas lysogenization with a mutant phage which has lost its suppressor function does not. Mating of *tsn*-K165 (*nal*^{r}) with strains 5349, 5351 and KH6 produced temperature resistant cells when selection was performed at 42°C on nalidixic acid plates. The temperature resistant cells presumably arose due to the insertion of the suppressor on the episomes. Control matings with strains KH17, 4258, and 4302 did not yield temperature resistant cells. These control matings indicate that the production of temperature resistant cells is not due to the adventitious insertion of the wild type allele of the temperature sensitive mutation carried in *tsn*-K165.

The reversion behavior of *tsn*-K165 supports its designation as a TSN mutant. Two types of temperature resistant colonies are obtained when selection for temperature resistant cells is carried out at 37°C. One type is *lac*$^+$ *trp*$^+$, presumably due to the spontaneous appearance of a temperature resistant suppressor. The other type of temperature resistant colony is *lac*^{ts} and *trp*^{ts} presumably because there is no change in the suppressor content of the cell. The original temperature sensitive suppressor leads to the phenotypic temperature sensitivity of the *lac* and *trp* mutations.

Table 3. Properties of temperature resistant and sensitive derivatives of *tsn*-K165

Strain	Growth at 42° C	*sup*$^+$ at 42° C	58K protein at 42°C
tsn-K165	−	n.d.	−
tsn-K165 (resistant to 42° C)	+	+	+
tsn-K165 (resistant to 37° C)	+/−[a]	+/−	+
tsn-K165 (ϕ80p*sup*III$^+$)	+	+	+
tsn-K165 (ϕ80p*sup*III$^-$)	−	n.d.	−
tsn-K165 (F′*supD*$^+$)	+	+	+
tsn-K165 (F′*supD*$^-$)	−	n.d.	−
tsn-K165 (F′141)	+	−	+
tsn-K165 (F′140)	+	−	+
tsn-K165 (F′-others)[b]	−	n.d.	n.d.

[a] There is generally a correlation between growth at 42° C and the presence of suppressor activity.
[b] A set of 16 other F′ containing strains that cover almost the entire genetic map.

At slightly higher temperatures (42°C) we find only one type of revertant which is *lac*$^+$ and *trp*$^+$ and therefore presumably *sup*$^+$. We do not yet understand the aberrant behavior of *tsn*-K165 at 42°C, except to speculate on the possibility that there may be two different suppressor sensitive mutations in the temperature sensitive cell, and at 42°C both must be suppressed in order for growth to occur.

Mapping the tsn-K165 Mutation

Interrupted matings with Hfr strains KL14 and BW113 gave temperature resistant recombinants. Further localization of the mutation was accomplished using episomes. From a set of 18 episomes containing strains which cover almost the entire genetic map, only two donors, 4248 and 4289, gave temperature resistant F-ductants. This indicates that the *tsn*-K165 mutation is localized between approximately 61 and 66 minutes on the *E. coli* genetic map.

Protein Composition of tsn-K165 at 42° C

When the proteins of strain *tsn*-K165 were analyzed by polyacrylamide gel electrophoresis as described in the Materials and Methods, it was found that at 42°C a major protein was not synthesized. This protein was found in the parental cells and in all other TSN and TSM mutants analyzed (Fig. 2, columns a and f, the arrow points to the affected protein). This protein is estimated to be the third or fourth most prominant protein as estimated by analysis of the autoradiograms on a Joyce-Loebl densitometer. The protein has a molecular weight of approximately 58000 as determined by the method of Weber and Osborn (1969). This protein is not a cell envelope protein as determined by the method of Chai and Foulds (1974) and is not present in the parental strains at lower temperatures. At 30°C it is almost entirely absent from both the parental and mutant cells and this temperature dependence appears in other bacterial strains as well (Cooper and Ruettinger, 1975). The 58000 MW protein synthesized and labeled in the parental cell at 42°C is not lost on subsequent incubation of the parental cell at 30°C in the absence of label.

Analysis of Temperature Resistant Derivatives of tsn-K165

When temperature resistant derivatives of *tsn*-K165 are analyzed for their protein composition at 42° C, we find that in all cases the 58000 molecular weight protein is present in the cells grown at 42° C (Table 3). This correlation of temperature resistance and the appearance of the missing protein holds for revertants, lysogens, and F-ductants. It does not matter whether the F′ donor inserts a suppressor function or the presumed wild type allele of the temperature sensitive mutation. Temperature sensitive cells obtained by curing the lysogens of their prophage or the F-ductants of their episomes (using acridine orange) do not produce the 58000 MW protein. Thus there appears to be a complete correlation between temperature sensitivity and the presence or absence of the 58000 MW protein at 42° C.

Analysis of ϕ80psupIII$^+$ Infected Cells

In addition to preparing stable temperature resistant lysogens with ϕ80p*sup*III$^+$ as described above, we have been able to demonstrate the suppressible nature of the synthesis of the 58000 MW protein in *tsn*-K165 by infecting the mutant at 30° C with a suppressor containing phage and demonstrating that subsequent incubation at 42° C allows synthesis of the protein (Fig. 2). Control infections with ϕ80p*sup*III$^-$ phage did not allow synthesis of the missing protein.

This result is presumed to occur by the infecting phage producing functional suppressor tRNA at 42° C which then acts to suppress the *tsn*-K165 mutation. This dynamic suppression is analogous to the production of β-galactosidase in *lac*$^-_{\text{am}}$ cells after infection with ϕ80p*sup*III$^+$ phage (Smith, Abelson, Clark, Goodman, and Brenner, 1966).

Growth of tsn-K165

In LB broth at 30° C *tsn*-K165 has a doubling time of 44–49 minutes, which is slightly slower than the growth rate of the parent SC122 (doubling time of 38 minutes). At 42° C the parent has a 24 minute doubling time while the turbidity of *tsn*-K165 increases for approximately 30 minutes and then stops. The turbidity does not double at 42° C. There is some slight but variable indication of cell lysis. Viability measurements indicate that the cells begin to lose the ability to form colonies at the time the turbidity stops increasing, and the viability decreases to about 1% viable cells over an hour.

Discussion

We have isolated a temperature sensitive cell which appears to contain a nonsense mutation in an essential function. The cell cannot grow at 42° C on broth medium unless a functional suppressor or the wild type allele of the cistron containing the temperature sensitive mutation is also present. We also find that the synthesis of a major protein is impaired in the mutant at 42° C. The protein is present in the temperature resistant derivatives of the mutant produced either by suppression or by insertion of the wild type allele of the mutant gene.

The cell presumably grows at 30° C because of the presence of a temperature sensitive suppressor which can function at the lower temperature. This pre-

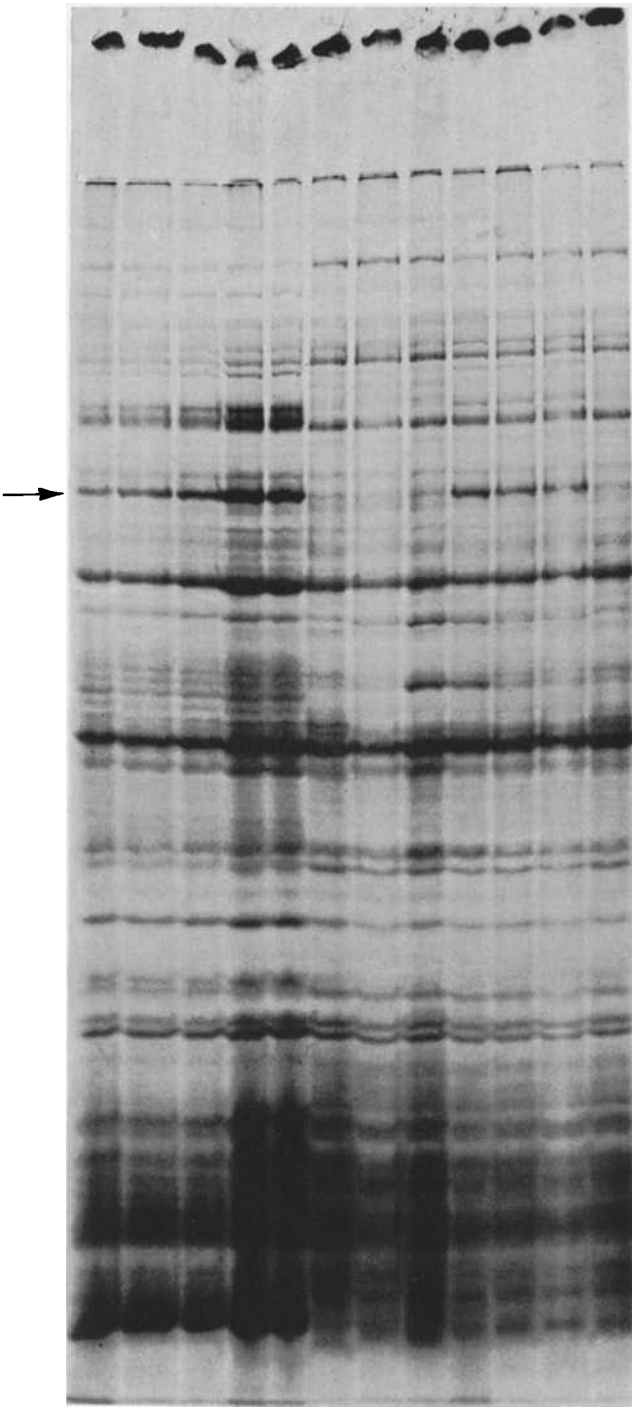

Fig. 2

sumption is slightly weakened by the fact that the particular protein (which is apparently affected by the nonsense mutation) is synthesized in very small amounts or not at all at 30° C (Cooper and Ruettinger, 1975).

The isolation of suppressor sensitive mutants has allowed the identification of many proteins with various structural genes in bacterial viruses. The isolation of *tsn*-K165 suggests that a similar approach may be feasible for essential functions in bacteria. Without the use of special techniques (Austin, Tittawella, Hayward, and Scaife, 1971, for example) the study of nonsense mutations in bacteria has been restricted to dispensible functions such as carbon source utilization or amino acid biosynthesis. As nonsense mutations are studied in bacterial viruses by using two different hosts for permissive and nonpermissive conditions, so two different temperatures can now be used to produce permissive and nonpermissive conditions for the identification and study of nonsense mutations in essential bacterial functions. A more detailed analysis of the potential of the TSN method is provided by Beckman and Cooper (1973).

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Fig. 2. Polyacrylamide gel electrophoresis of total protein from cells infected with phage ϕ80p*sup*III$^+$ and ϕ80p*sup*III$^-$. SC122 and *tsn*-K165 were grown to a concentration of $1–2 \times 10^8$ cells/ml at 30° C in glucose minimal medium supplemented with all amino acids except leucine and valine. At different times prior to shifting the cells from 30° C to 42° C, 5.0 ml volumes of the cultures were infected with a multiplicity of 10 pfu per cell. Fifteen minutes after the shift to 42° C the cultures were labeled for 15 min with ^{14}C-valine. At the end of the labeling period cold TCA (5% final concentration) was added and the cells were collected by centrifugation and analyzed as described in the Material and Methods

	Strain	Treatment	Min prior to shift to 42° C
a	SC122	uninfected	
b	SC122	ϕ80p*sup*III$^+$	15
c	SC122	ϕ80p*sup*III$^-$	15
d	SC122	ϕ80p*sup*III$^-$	35
e	SC122	ϕ80p*sup*III$^+$	35
f	*tsn*-K165	uninfected	
g	*tsn*-K165	ϕ80p*sup*III$^-$	15
h	*tsn*-K165	ϕ80p*sup*III$^-$	35
i	*tsn*-K165	ϕ80p*sup*III$^+$	35
j	*tsn*-K165	ϕ80p*sup*III$^+$	25
k	*tsn*-K165	ϕ80p*sup*III$^+$	15
l	*tsn*-K165	ϕ80p*sup*III$^+$	5

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Dr. Stephen Cooper
Therese Ruettinger
Department of Microbiology
University of Michigan
School of Medicine
Ann Arbor, Michigan 48103
USA