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A delicate relationship between ppGpp and UPRTase in uracil uptake in *Escherichia coli*

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Summary

Under amino acid starvation, when the intracellular level of guanosine tetraphosphate (ppGpp) is high, uracil uptake of *Escherichia coli* is strongly decreased according to some early studies. However, some researchers say that uracil phosphoribosyltransferase (UPRTase), which is involved in the uptake of uracil and its conversion to uridine 5'-monophosphate (UMP), is activated by ppGpp *in vitro*. Here, I have studied the mechanism of uracil uptake in different *Escherichia coli* strains that carried different alleles of *valS* (encodes valyl-tRNA synthetase), *uraA* (uracil permease) and *relA* (ppGpp synthetase). ppGpp accumulated in *valS*^{ts} mutants but not in the *valS*^{ts}, *relA* mutant when the temperature rose. Growth experiments with these mutants were implemented in different media at different temperatures. The original pyrimidine-requiring strain *SØ1259* had the shortest generation time at all temperatures and media, while its *valS*^{ts} mutant *ML100* had a longer generation time at 33.5℃ than at 30℃, and longer in minimal medium with casamino acid than in rich medium Luria–Bertani broth. The stringent response (production of ppGpp stimulated at a higher temperature in *valS*^{ts} mutants) indeed had an inhibitory effect on uracil uptake of the *valS*^{ts} pyrimidine-requiring mutant (*ML100*). However, while ppGpp had a down-regulatory effect on exogenous uracil uptake, the interference of *valS*^{ts} mutation itself, the composition of media and the turnover of nucleotide pools should be considered by testing different media and uracil concentrations in growth or incorporation experiments. UraA was not necessary for uracil uptake at a high exogenous uracil concentration after all, but the function of UraA on regulation of uracil uptake is still unclear. No evidence indicated that ppGpp activated uracil uptake.

Introduction

Uracil uptake

There are two pathways to synthesize uridine 5'-monophosphate (UMP) in *Escherichia coli*. One is the *de novo* pathway, and another is the *salvage* pathway (**Figure 1**). Some of the enzymes that catalyze steps in each pathway and the genes encoding these enzymes were described by Jensen *et al*. (1984), e.g. OMP decarboxylase encoded by *pyrF*, aspartate transcarbamylase by *pyrB*, uridine kinase by *udk* and uracil phosphoribosyltransferase by *upp*. Elimination of any one of these enzymes in the branch (**Figure 1**) shuts down the pathway. For example, if the cells have a knockout mutation in the gene *pyrF*, the *de novo* pathway is shut down and UMP is only synthesized through the *salvage* pathway.

Uracil uptake is the process in which exogenous uracil is transported and converted into intracellular UMP which later becomes substrates (UTP, CTP) for RNA polymerization. It is known that uracil phosphoribosyltransferase (UPRTase for short) catalyzes the transfer of ribose phosphate from PRPP to uracil to form UMP. It is also necessary for uptake of uracil in *E. coli* (Nyhan 2005 and Munch-Petersen and Mygind 1983).

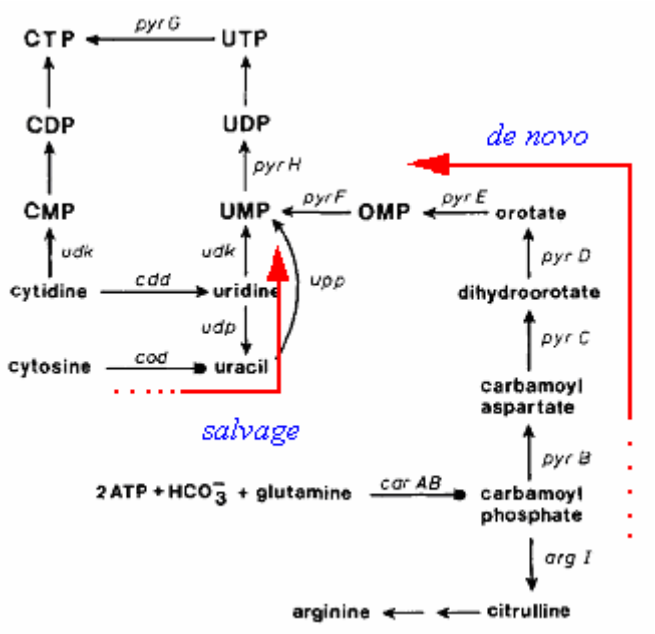

Figure 1. Pathways for the biosynthesis of pyrimidine nucleoside triphosphates in *E. coli*. The branch where the *pyrF* on is the *de novo* pathway; the branch where uracil is converted into UMP by UPRTase (*upp*) is the *salvage* pathway. Reproduced from Jensen *et al*. 1984 with permission from the copy right owner.

ppGpp and the stringent response

ppGpp, which is a global regulator of gene expression and thus has effects on cell physiology (Magnusson *et al*. 2005), is produced in response to uncharged tRNA (carrying no amino acid) in the ribosomal A-site under amino acid starvation. Increased levels of ppGpp finally lead to a reduction in synthesis of ribosomal and transfer RNAs (stable RNAs). The dramatic down-regulation of biosynthesis of stable RNAs under amino acid starvation is known as the stringent response and growth conditions like amino acid starvation that arouse the stringent

response are called stringent conditions.

An inhibitory effect of ppGpp on uracil uptake?

Many early investigations showed that the incorporation of exogenous uracil into stable RNAs of *E. coli* is strongly inhibited under stringent conditions (Edlin and Neuhard 1967, Fast and Sköld 1977, Fast 1978). Some researchers (Fast and Sköld 1977 and Kern *et al*. 1990) suggested that ppGpp has a direct inhibitory effect on UPRTase. However, in a recent study (Jensen and Mygind 1996) could not detect any inhibitory effect of ppGpp on pure UPRTase when Mg^{2+} was in excess *in vitro*. A uracil permease, UraA, which resides on the cytoplasmic membrane, was found to be necessary for *E. coli* to take up uracil at low exogenous uracil concentrations even at high UPRTase activity (Andersen *et al*. 1995).

Mutations affecting uracil uptake and metabolism

1. Cells growth relies on exogenous uracil

E. coli that carries a Δ*pyrF* mutation is unable to synthesize UMP by the *de novo* pathway (**Figure 1**) and therefore depends upon the *salvage* pathway (Jensen *et al*. 1984). This means that a pyrimidine-requiring mutant will not survive if the cells are unable to take up uracil from the medium.

2. Cells ppGpp level is regulated by temperature

A strain that carries a *valS*^{ts} mutation encodes a temperature sensitive valyl-tRNA synthetase. At a restrictive temperature (>30℃), the uncharged valyl-tRNA accumulates since the tRNA aminoacylation is inhibited (Patrick and Masayasu 1974, Heck and Hatfield 1988). When the uncharged valyl-tRNA gets into the ribosomal A-site, protein synthesis will stop since the uncharged valyl-tRNA is unable to move to the P-site and other amino acids can't get into the A-site to connect to the growing polypeptide chain. At the same time, since the uncharged valyl-tRNA is in the ribosomal A-site, ppGpp will be produced just like under stringent conditions mentioned above. Thus, if a mutant has both *valS*^{ts} and Δ*pyrF* mutations, the generation time of this mutant at different temperatures represents the efficiency of uracil uptake and furthermore the inhibitory effect of ppGpp on uracil uptake.

3. UraA or ppGpp insufficiency mutants

An *uraA* mutant has an abnormal UraA (uracil permease) or no UraA on the cytoplasmic membrane, but it still has a normal UPRTase (uracil phosphoribosyltransferase) activity. The pyrimidine-requiring *uraA* mutant grows more slowly than the parent at a low concentration of uracil (9 mM), but grows like the parent at 90 mM uracil, and takes up uracil to the same extent as the parent strain and incorporates uracil into nucleic acids at the same rate (Andersen *et al*. 1995).

A *relA* mutant has no ppGpp or stringent response under stringent conditions, so it is also called the relaxed mutant. Usually, the synthesis of stable RNAs (mainly rRNA and tRNA) is swiftly shut down in the *rel*$^{+}$ strain under stringent conditions, with the intracellular accumulation of ppGpp. However, in the relaxed mutant (*rel*$^{-}$), stable RNAs synthesis continues for a long time even under amino acid starvation (Burkovski *et al*. 1995, Fast 1978, Magnusson *et al*. 2005, Srinivasan *et al*. 1973 and Uzan and Danchin 1976).

Aims

Based on inconsistent experimental results *in vivo* and *in vitro*, the uncertain effect of ppGpp on uracil uptake and unclear mechanisms of uracil uptake in *E. coli*, I aimed to learn more about uracil uptake under stringent conditions, the effect of ppGpp on uracil uptake or the UPRTase and the role of UraA in uracil uptake. My result suggested that ppGpp had a down-regulatory effect on the uptake of exogenous uracil.

Results

Uracil uptake, ppGpp and UraA in *E. coli SØ1259*

1. Initial characterization of strains

In order to analyze the possible role of ppGpp in uracil uptake, I started from a pyrimidine-requiring strain *SØ1259* (Δ*pyrF*) and constructed a strain where I could control the stringent response. As mentioned before, the growth rate of the pyrimidine-requiring mutant is a measure of its efficiency of uracil uptake. I moved a *valS*^{ts} allele into *SØ1259* by P1 cotransduction with the neighboring *zjh*::Tn*10* allele, selected for tetracycline resistance and screened for the *valS*^{ts} transductant by incubation at 37℃; a *valS*^{ts} mutant did not show any growth at all after 24h incubation. The stringent response of the *SØ1259 valS*^{ts} mutant named *ML100* (Δ*pyrF*, *valS*^{ts}) could now be regulated by the temperature. As a control, I constructed a non-pyrimidine-requiring *valS*^{ts} mutant named *ML101* (*pyrF*$^{+}$, *valS*^{ts}), the growth of which did not depend upon exogenous uracil by moving a wild type *pyrF* allele into *ML100* followed by selection on a minimal plate without uracil.

SØ1259 did not require any exogenous amino acid and grew quite well in minimal medium (M9) with uracil. However, when the *valS*^{ts} allele had been transferred into *SØ1259*, *valS*^{ts} mutants failed to grow in minimal medium whether uracil was added or not. I tried adding valine and/or isoleucine that share the same metabolic pathway as valine, since *valS*^{ts} strains are easily starved for valine, but found that M9 with casamino acids (CAA), was the only medium in which *valS*^{ts} mutants grew normally (**Table 1**). A more precise growth experiment was also carried out with *ML100* and *SØ1259*, which demonstrated that the *valS*^{ts} mutant *ML100* failed to grow in minimal medium with uracil (or uridine), while the parent *SØ1259* grew normally (**Figure 2**). In contrast, both of them grew well in minimal medium with CAA and uracil, and their generation times were 50 minutes for *ML100* and 60 minutes for *SØ1259* at 30℃. The reason for the auxotrophy of the *valS*^{ts} strain is unknown.

In the second step, I constructed a pyrimidine-requiring *uraA* mutant (Δ*pyrF*, *valS*^{ts}, *uraA*), the recipient *ML100* (*valS*, *zjh*::Tn*10*) first had to go through the Bochner selection, which utilized the fact that tetracycline resistant cells are hypersensitive to fusaric acid (Bochner *et al*. 1980). A fusaric acid resistant *ML100* candidate, from which the Tn*10* transposon had been eliminated spontaneously, was then verified to be tetracycline sensitive. Then *uraA*::Tn*10* was transduced into that *ML100* candidate, and a tetracycline resistance transductant named *ML102* was selected and purified.

Finally, a pyrimidine-requiring *relA* mutant named *ML110* (Δ*pyrF*, *relA*) was constructed by transducing a *relA* allele into *SØ1259* by cotransduction with the neighboring *zga*::Tn*10kan* allele. This selection was based on inability of *relA* strains to grow in minimal medium M9 supplemented with L-serine, L-methionine and L-glycine (Uzan and Danchin 1976), where *relA*$^{+}$ strains grew normally well. However, it was not possible to construct a *valS*^{ts} *ML110*, since neither a *valS*^{ts} *relA*$^{+}$ strain (*ML100*) nor a *valS*^{ts} transductant of *ML110* grew up on the selection plates used. Growth of all strains is shown in **Table 1**.

Table 1. Growth of pyrimidine-requiring mutants in different media[a]

		Strain (genotype)				
		SØ1259	*ML100*	*ML101*	*ML102*	*ML110*
Temp℃	Medium	(Δ*pyrF*)	(Δ*pyrF valS*^{ts})	(*pyrF*$^{+}$ *valS*^{ts})	(Δ*pyrF valS*^{ts} *uraA*)	(Δ*pyrF relA*)
25	M9+ura[b]	+	-	-	-	+
	M9+CAA[c]+ura	++	+	+	+	+
	M9+val+ile	+	-	-	-	+
	M9+val	+	-	-	-	+
	M9+ile	+	-	-	-	+
30	M9+ura	+	-	-	-	+/-?
	M9+CAA+ura	++	+	+	+	+
	M9+val+ile	+	-	-	-	+/-?
	M9+val	+	-	-	-	+/-?
	M9+ile	+	-	-	-	+/-?

[a] Cells were incubated separately overnight in different media at 25℃ or 30℃. M9+val+ile, M9+val or M9+ile was supplemented with uracil when culturing Δ*pyrF* mutants. Next day, the growth was estimated visually, '++', strong growth, '+', some growth, '-', no growth, and '+/-?' data were ambiguous.

[b] Uracil (ura) final concentration was 10 μg/ml.

[c] Casamino acids (CAA) final concentration was 0.2%.

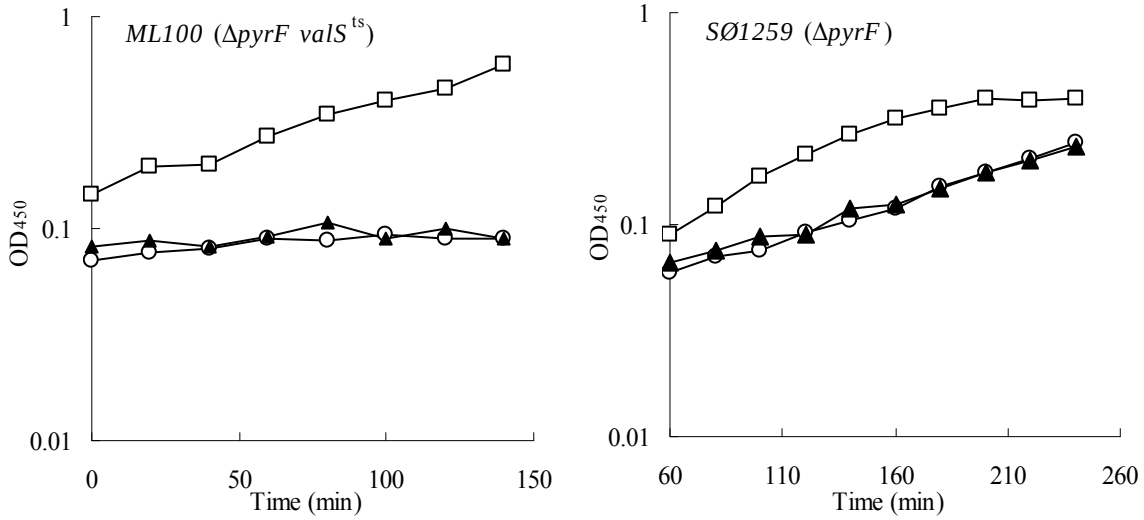

Figure 2. Comparison of the growth in different media. Cells were incubated separately in three different media at 30℃, (□) M9 with casamino acids and uracil; (○) M9 with uracil; (▲) M9 with uridine. 1 ml sample was taken out every 20 minutes and its OD_{450} was measured in a spectrophotometer. An approximate generation time was calculated from the growth curve.

Combining all strains I had constructed, a series of growth experiments were carried out to demonstrate the effect of stringent regulation on the growth of pyrimidine-requiring mutants.

2. Effect of stringent regulation on the growth of E. coli SØ1259

In order to obtain more information about uracil uptake under stringent conditions and the regulatory effect of ppGpp on uracil uptake, growth experiments were carried out with different *valS*^{ts} pyrimidine-requiring mutants at different temperatures. As mentioned before, the stringent response or the production of ppGpp was stimulated by an increased temperature, so differences in the generation time of the *valS*^{ts} pyrimidine-requiring mutant at 30℃, 33.5℃ or 37℃ represented some effect of stringent regulation on uracil uptake in *E. coli*. On the other hand, *valS*^{ts} pyrimidine-requiring mutants only grew in M9 if casamino acids (CAA) were added, so growth experiments in rich medium (Luria–Bertani broth, LB), which provides many different carbon and energy sources including sugars, alcohols, and organic acids (Beav *et al*. 2006), were performed as controls and a comparison of growths in these two media would reveal an interfering effect of media on the stringent regulation of uracil uptake.

All strains were incubated in M9 with CAA, or LB, at different temperatures. **Table 2** shows the generation times that were calculated from the growth curves (**Figure 3**). In M9 with CAA, *valS*^{ts} mutants *ML100*, *ML101* and *ML102* all grew more slowly than the parent *SØ1259* at 30℃, *ML100* (Δ*pyrF valS*^{ts}) had a shorter generation time than *ML101* (*pyrF*$^{+}$ *valS*^{ts}) at 30℃, but, visible longer at 33.5℃, and both of them had longer generation times at 33.5℃ than at 30℃. This suggested that the stringent response down-regulated the growth, possibly by inhibiting uracil uptake.

The temperature effect on growth of the *uraA* mutant *ML102* (Δ*pyrF valS*^{ts} *uraA*) was similar to the effect on its *uraA*$^{+}$ parent *ML100* at 30℃ or 33.5℃. Thus, the elimination of UraA in *SØ1259* was inconsequential for effects of temperature on uracil uptake when the concentration of uracil in M9 with CAA was 10 μg/ml. The *relA* mutant *ML110* (Δ*pyrF relA*), which was not temperature sensitive, grew as well as the parent *SØ1259* at 30℃. However, when the temperature rose, generation times of *ML110* became longer while the parent had a shortest one at 37℃.

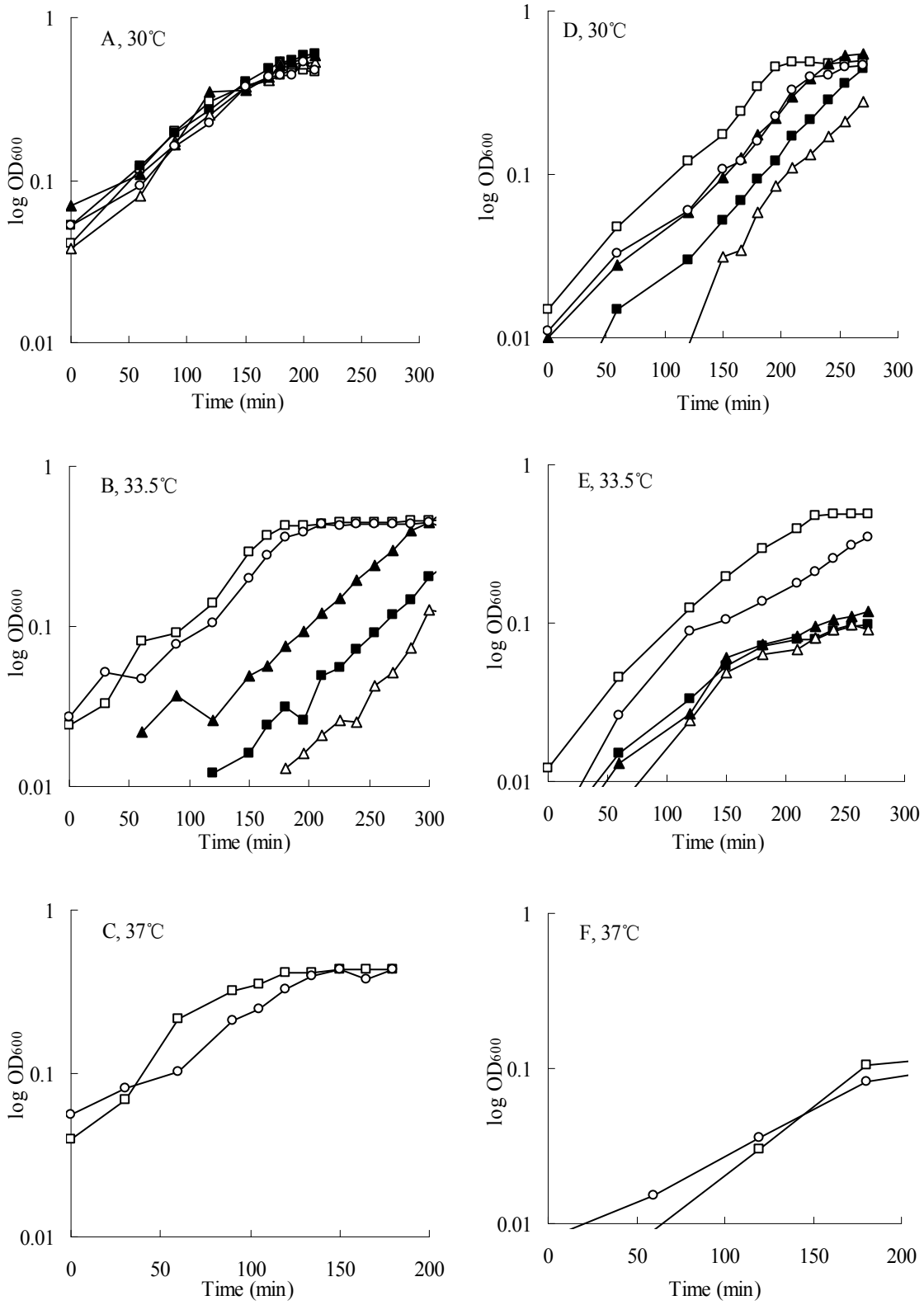

Figure 3. Effects of the stringent regulation and media on the growth of *SØ1259* (Δ*pyrF*, □), *ML100* (Δ*pyrF valS*^{ts}, ■), *ML101* (*pyrF*$^{+}$ *valS*^{ts}, △), *ML102* (Δ*pyrF valS*^{ts} *uraA*, ▲) and *ML110* (Δ*pyrF relA*, ○). Strains were incubated separately in LB (A-C) or M9 with casamino acids (CAA) and uracil (10 μg/ml) (D-F) at different temperatures. 1 ml sample was taken out every 10 or 20 minutes and its OD_{600} was measured in a spectrophotometer. The scale on the y axis is logarithmic. *when *ML101* was incubated in M9 with CAA, no uracil was added and *ML110* was always cultivated with kanamycin.

Table 2. Generation times of *E. coli SØ1259*[a]

		Strain				
Medium	Temp℃	*SØ1259* (Δ*pyrF*)	*ML100* (Δ*pyrF valS*^{ts})	*ML101* (*pyrF*$^{+}$ *valS*^{ts})	*ML102* (Δ*pyrF valS*^{ts} *uraA*)	*ML110* (Δ*pyrF relA*)
M9+CAA[b]	30[c]	60	72	84	78	60
	33.5	45	171[e]	136.5[e]	177[e]	84
	37	42	—[d]	—	—	78
LB	30	38.3	46.5	34.5	51	42
	33.5	27	42	36	45	33
	37	18	—	—	—	29.5

[a] The data were calculated from growth curves shown in **Figure 3**

[b] Uracil was added to M9 with casamino acids when incubating Δ*pyrF* strains.

[c] Generation time was in minute.

[d] Cells had no growth.

[e] The value of generation time may be imprecise.

In summary, the stringent response (production of ppGpp stimulated at a higher temperature in *valS*^{ts} mutants) indeed caused the *valS*^{ts} Δ*pyrF* mutant (*ML100*) to grow more poorly than its Δ*pyrF* parent (*SØ1259*) or the *valS*^{ts} *pyrF*$^{+}$ mutant (*ML101*) in M9 with CAA and uracil at a permissive temperature. In rich medium, *valS*^{ts} strains grew faster at 30℃ and 33.5℃, but not at 37℃. This suggested that abundant amino acids in LB can alleviate some amino acid starvation of *valS*^{ts} mutants at permissive temperatures but not at restrictive temperatures (≥37℃).

Uracil uptake, ppGpp and UraA in *E. coli RH491*

E. coli RH491 is an auxotroph and needs leucine, lysine, histidine, valine, isoleucine and tryptophan to grow. Since it is an auxotroph, *RH491* was easily starved for amino acid and became more sensitive to the stringent conditions. Thus, investigating the effect of stringent regulation on uracil uptake in *RH491* and its derivatives would provide certain information concerning uracil uptake under stringent conditions which wasn't obtained from the study of *E. coli SØ1259*.

1. Initial characterization of strains

I started from a *valS*^{ts} pyrimidine-requiring strain *ML200* which had been constructed from *RH491* by Robert Fast. As mentioned before, the growth rate of pyrimidine-requiring mutants can represent their efficiency of uracil uptake. As a control, I constructed a non-pyrimidine-requiring *valS*^{ts} mutant named *ML201* by moving a wild type *pyrF* allele into *ML200* and selecting on a minimal medium without uracil. Because the parent *RH491* had several amino acid requirements, the minimal medium for *ML200*, *ML201* and other *RH491* derivatives was always supplemented with required amino acids or casamino acids plus tryptophan.

A *valS*^{ts} pyrimidine-requiring *relA* mutant (Δ*pyrF*, *valS*^{ts}, *relA*) was constructed by moving a *relA* allele into *ML200* by cotransduction with the neighboring *zga*::Tn*10kan* allele, selecting for kanamycin resistance (Kan^{R}) and screening those transductants for the *relA* allele (Uzan and Danchin 1976). Since this screening did not take the *valS*^{ts} mutation into consideration the presence of *relA* allele in the studied strain named *ML210** was uncertain. However, as mentioned before, the synthesis of stable RNAs (mainly rRNA and tRNA) is swiftly shut down in *rel*$^{+}$ strain under stringent conditions, with the intracellular accumulation of ppGpp, but in the relaxed mutant (*rel*$^{-}$), the synthesis of stable RNAs continues for a long time during amino acid starvation (Burkovski *et al*. 1995, Fast 1978, Magnusson *et al*. 2005, Srinivasan *et al*. 1973 and Uzan and Danchin 1976). Therefore, I used ^{14}C-uridine incorporation (Fast 1978 and Srinivasan *et al*. 1973) to track the synthesis of stable RNAs of *ML210** to test the presence of the *relA* allele, using two methods: direct amino acid starvation (**Table 3**) and indirect starvation by growing the *valS*^{ts} mutant at a restrictive temperature (**Figure 4**). The incorporation of ^{14}C-uridine into acid-insoluble material was estimated by precipitation with trichloroacetic acid. However, no clear-cut results were obtained in the incorporation experiments.

1.1 Direct amino acid starvation.

*ML210** was incubated in minimal medium (M9) with ^{14}C-uridine but lacking source of required amino acids leucine, lysine, histidine or tryptophan. As a control, a well-known *rel*$^{-}$ strain *CP79*, which was auxotrophic and required arginine, threonine, leucine and histidine, was also included in a similar experiment. Results in **Table 3** showed very low ^{14}C-uridine incorporation by *ML210** in M9 only supplemented with valine and isoleucine, but high incorporation in M9 with all required amino acids. *CP79* showed high incorporation in the medium (M9+aa) lacking arginine and threonine or the medium with casamino acids. *ML200* (*rel*$^{+}$) and *CP78* showed similar incorporation as *ML210**. Therefore, *ML210** did not behave as a true *relA* strain in this experiment.

Table 3. Incorporation of ^{14}C-uridine into stable RNA under amino acid starvation[a]

	Strain			
Medium	*ML210** (*relA*$^?$)	*ML200* (*relA*$^+$)	*CP79* (*rel*$^-$)	*CP78*(*rel*$^+$)
M9+val+ile	**263**	722	ND	ND
M9+aa[b]+trp	**1559**	2137	ND	ND
M9+aa	ND[c]	ND	**1637**	608
M9+CAA	ND	ND	**1716**	1284
Blank[d]	38	36	93	65

[a] Cells were incubated in different media. After 45 minutes incubation, ^{14}C-uridine was added into each medium. After 30 minutes ^{14}C-uridine incorporation, TCA-precipitable radioactivity of each sample was estimated. The data shown in the table are CPM values.

[b] Required amino acids: L-leucine, L-lysine, L-histidine, L-valine and L-isoleucine.

[c] The incorporation was not determined.

[d] Blank samples were no addition of ^{14}C-uridine.

1.2 Indirect amino acid starvation.

ValS^{ts} strains were incubated at restrictive temperatures. When the incubation temperature was raised (to 37℃ or 42℃), the protein synthesis was halted in *valS*^{ts} strains as if they were starving for valine, and ppGpp was produced in response to the uncharged valyl-tRNA. The synthesis of the stable RNAs then would be shut down immediately by the ppGpp in *rel*$^+$ strains but not in *rel*$^-$ one. According to **Figure 4**, the incorporation of ^{14}C-uridine into stable RNAs in *ML210** was increased along with time even when the incubation temperature was raised to 37℃ or 42℃. Unexpectedly, *ML200* (*rel*$^+$) also behaved this way. Since time was limited in my project, I didn't continue to screen other *relA* candidates and just used *ML200*, *ML201* and the *valS*^{ts} pyrimidine-requiring unsure *relA* mutant *ML210** in later growth experiments to obtain more data concerning effects of stringent regulation on growth of pyrimidine-requiring mutants and uracil uptake.

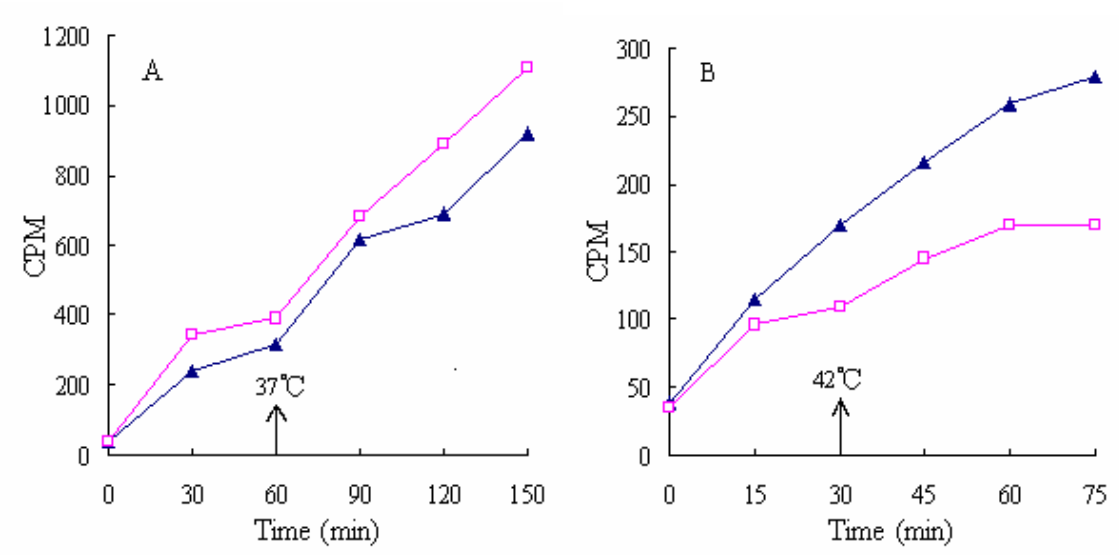

Figure 4. Incorporation of ^{14}C-uridine into stable RNA. In diagram A, cells of *ML200* (▲) and *ML210** (□) were first incubated at 30℃ in minimal medium (M9) with required amino acids. At the zero point on the graph,

^{14}C-uridine was added into the culture and the incubation temperature was shifted to 37℃ at 60 minutes. In diagram B, the incubation temperature was shifted to 42℃ at 30 minutes on the graph. The TCA-precipitable radioactivity (CPM) of each sample was estimated by procedures mentioned before.

2. The effect of stringent regulation on the growth of E. coli RH491

According to the results obtained from *E. coli SØ1259*, the stringent response had some effect on uracil uptake in *valS*^{ts} Δ*pyrF* mutants. However, the *valS*^{ts} mutation itself also had an inhibitory effect on the growth of cells, as neither the pyrimidine-requiring *valS*^{ts} mutant nor the non-pyrimidine-requiring *valS*^{ts} mutant grew at 37℃. 37℃ (or higher) was restrictive to *SØ1259 valS*^{ts} mutants. In order to investigate whether *RH491 valS*^{ts} mutants *ML200* and *ML201* behaved differently at restrictive temperatures, a preliminary experiment was carried out where bacterial cultures in tubes were grown at different temperatures overnight. The results (**Figure 5**A) showed that neither the pyrimidine-requiring mutant (*ML200*) nor the non-pyrimidine-requiring mutant (*ML201*) grew at 36℃, 39℃ or 42℃. All evidence indicated that the growth suppression caused by *valS*^{ts} mutation wasn't reversed by *pyrF*$^{+}$ allele. On the other hand, the *salvage* pathway was considerably more energy-efficient than the *de novo* pathway (Nyhan 2005), Δ*pyrF* and *pyrF*$^{+}$ strains should grow similarly in complete media with uracil at 30℃ according to results of *E. coli SØ1259*, but *ML201* (*pyrF*$^{+}$ *valS*^{ts}) grew much more and faster than *ML200* (Δ*pyrF valS*^{ts}) at 30℃ (**Figure 5**B). This suggested that there was some kind of inhibition of uracil uptake or of the activity of UPRTase of *ML200* even at a permissive temperature (30℃).

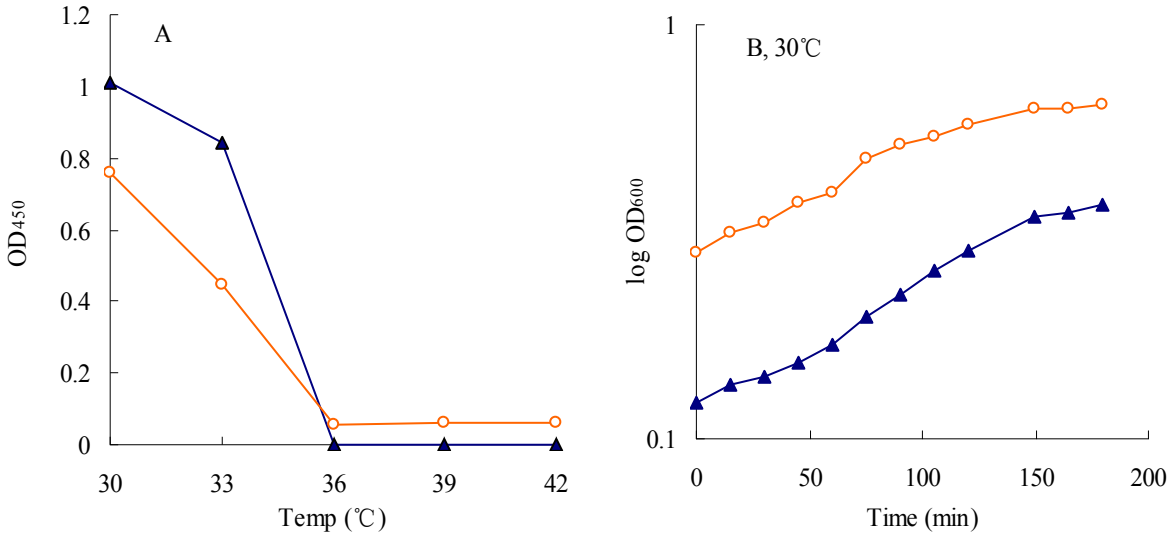

Figure 5. Growth of *ML200* (▲) and *ML201* (○). A, Cells were incubated overnight in minimal medium with casamino acids, L-tryptophan and uracil (M9+CAA+trp+ura) at different temperatures. Next day, OD_{450} of the cultures was measured. B, Cells were grown at 30℃. *ML200* was cultivated in M9+CAA+trp+ura; *ML201* was cultivated in M9+CAA+trp. The scale on the y axis in B is logarithmic.

Since these *RH491 valS*^{ts} mutants looked similar to *SØ1259 valS*^{ts} mutants in these growth experiments, a different experimental strategy was adopted to analyze the stringent regulation of uracil uptake.

*3. Effect of uracil concentration on the putative relA mutant ML210**

In order to test the mutant *ML210** in uracil uptake under stringent conditions, *ML200* and *ML210** were incubated overnight with different amount of uracil at 30℃ and 33.5℃. Different amounts of uracil were added into the same volume of bacteria culture. The OD_{600} of overnight cultures represent their cell mass. As seen in **Figure 6**, *ML200* and *ML210** grew to lower cell densities at 33.5℃ than 30℃ at all uracil concentrations. When uracil concentration was higher than 50 μg/ml, the cell mass of *ML210** was much lower than that of *ML200* at 33.5℃ while they were very close at 30℃. All this suggested that ppGpp may be necessary for *E. coli* to regulate uracil uptake. However, because of the uncertain genotype of *ML210**, it is necessary to screen out a true *relA* mutant and repeat the same experiment.

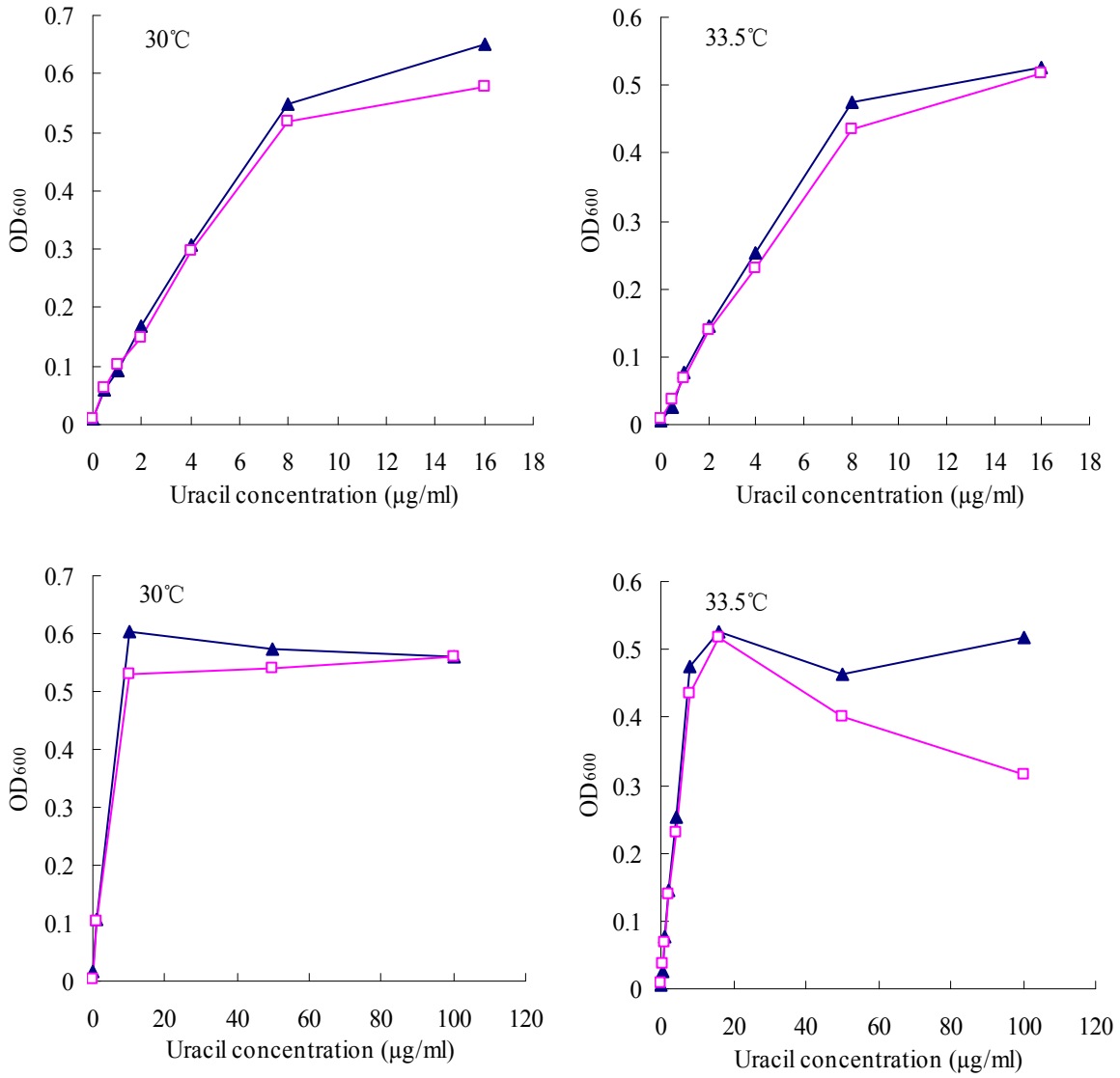

Figure 6. Uracil uptake of *relA* strain at different temperatures. Cells of *ML200* (▲) and *ML210** (□) were incubated overnight in minimal medium (M9) with required amino acids and different amounts of uracil at 30℃ or 33.5℃. Next day, OD_{600} was measured in a spectrophotometer.

Discussion

The experiments reported here address the mechanism of uracil uptake in *E. coli* and the roles of ppGpp or UraA in the uracil uptake system.

ppGpp has an inhibitory effect on uracil uptake

1. Growth was inhibited at semi-permissive temperatures

Shifting the *valS*^{ts} cultures from a permissive growth temperature to a semi-permissive growth temperature can cause a partial restriction of valyl-tRNA aminoacylation and protein synthesis that elicits the stringent response in *rel*$^{+}$ strains (Patrick 1975). The growth of the *valS*^{ts} pyrimidine-requiring mutants are dependent upon uptake of exogenous uracil. A comparison of the growth of the *valS*^{ts} pyrimidine-requiring mutant *ML100* and the *valS*^{ts} non-pyrimidine-requiring mutant *ML101* (**Table 2**) demonstrated that uracil uptake was partially inhibited at a semi-permissive temperature (33.5℃) and the stringent response had a down regulatory effect on uracil uptake. The mechanism of stringent regulation of ppGpp, which accumulates in response to several stress conditions, including nutrient limitation and amino acid starvation, is still controversial and most models include both direct and indirect effects on cell gene expression and physiology (Magnusson *et al*. 2005). My results as well as some old results in this laboratory (Fast and Sköld 1977) suggested that ppGpp had an inhibitory effect on uracil uptake. Moreover, some kind of inhibition of uracil uptake at a permissive temperature (30℃) of another *valS*^{ts} pyrimidine-requiring mutant *ML200* (**Figure 5**B), which was auxotrophic and elicited the stringent response more easily, suggested that the down-regulating effect of ppGpp was stronger in an amino acid requiring strain.

2. Rich media may alleviate the inhibitory effect of ppGpp

ValS^{ts} mutants might have a higher intracellular level of ppGpp than the parent even at a permissive temperature, as *SØ1259 valS*^{ts} mutants were unable to grow in the minimal medium M9 while the parent grew normally (**Table 1** and **Figure 2**), and *valS*^{ts} mutants grew more slowly than the parent at 30℃ in the minimal medium M9 with casamino acids (CAA) or in the rich medium LB (**Table 2**). However, the high concentration of amino acids and other nutrients in CAA or LB seemed to counterbalance the high level of ppGpp and quench the stringent response in *valS*^{ts} mutants even at a semi-permissive temperature (33.5℃) but not at a restrictive temperature (37℃). *ValS*^{ts} mutants grew much faster in LB than in M9 with CAA, and had significant differences in generation times at 30℃ and 33.5℃ in M9 with CAA while they did not show any remarkable difference in the rich medium LB (**Table 2**). This also demonstrated that the inhibitory effect on uracil uptake only appeared under stringent conditions with the production of ppGpp (Edlin and Neuhard 1967, Fast and Sköld 1977 and Fast 1978).

3. Interference caused by the valS^{ts} mutation itself and ppGpp

The *valS*^{ts} mutation itself had some inhibitory effects on the growth and caused auxotrophy in the *valS*^{ts} strain, as demonstrated by the observation that *SØ1259 valS*^{ts} mutants did not grow in minimal medium (**Table 1** and **Figure 2**). Moreover, the *valS*^{ts} mutant *ML100* and the parent *SØ1259*, both of which should have a similar efficiency in uracil uptake at a permissive

temperature, grew differently in LB or in M9 with CAA and uracil at 30℃ (**Table 2**). On the other hand, ppGpp, the production of which was stimulated by the *valS*^{ts} mutation may be produced even at a permissive temperature, as growth of *ML200* seemed to be suppressed even at 30℃ (**Figure 5**B). The regulatory effects of ppGpp on gene expression or cell physiology (Magnusson *et al*. 2005) beside uracil uptake may relate to the suppression of the growth of *valS*^{ts} mutants. So differences in the growth may not only represent the inhibitory effect on the uptake of uracil, but also some other effects of the *valS*^{ts} mutation itself and ppGpp on cell growth. Both these seemed to induce a more unpredictable interference on growth experiments results that were supposed to demonstrate stringent regulation of ppGpp on uracil uptake. It is likely that the interference might become more and more severe as the temperature is raised. However, this kind of interference should be the same in *valS*^{ts} pyrimidine-requiring mutants (*ML100*, *ML200*) and *valS*^{ts} non-pyrimidine-requiring mutants (*ML101*, *ML201*), and neither of them grew at a restrictive temperature (≥37℃) (**Table 2** and **Figure 5**A). Based on the difference in the growth of these two mutants, it still appears that ppGpp has an inhibitory effect on uracil uptake. Growth experiments that are carried out at a temperature lower than 30℃ may give more information about the impact of the *valS*^{ts} mutation and the production of ppGpp.

ppGpp may inhibit uracil phosphoribosyltransferase directly

At a low exogenous uracil concentration (9 μM), UraA is necessary for uracil uptake (Andersen *et al*. 1995). However, in order to obtain comparable growth results of different mutants, I used a higher uracil concentration, 10 μg/ml (90 mM). Here, the *uraA* mutant *ML102*, which had a normal uracil phosphoribosyltransferase (UPRTase) activity, showed the same pattern of growth and uracil uptake as its *ML100* parent. Since UPRTase (encoded by *upp*) is the most important enzyme involved in uracil uptake (Kern *et al*. 1990 and Andersen *et al*. 1995), the inhibitory effect of ppGpp on uracil uptake should be exerted mainly on the UPRTase, meaning that UraA is dispensable for uracil uptake at a high exogenous uracil concentration. There is no sign indicating that ppGpp activates uracil uptake under stringent conditions *in vivo*.

Unstable RNAs and turnover of nucleotide pools should be considered in incorporation experiments

The inconsistent results in the incorporation experiments when screening for a real *relA* mutant may be due to many reasons. Stable RNAs (tRNA and rRNA) should be largely reduced in growing cells under stringent conditions. However, a considerable synthesis of unstable RNA, which is labeled by isotopes but has a very short life, still exists (Nierlich 1968). So the unexpected increase of incorporation in *ML200* (**Figure 4**) may due to the reuse of degradation products (radioactive precursors) of the unstable RNA in stable RNA synthesis (Nierlich 1968). In the direct amino acid starvation incorporation experiment, the unsure *relA* mutant *ML210** was incubated in M9 only with valine and isoleucine but no uracil or uridine. The cells were under severe amino acid starvation and lacked uracil or uridine to synthesize the pyrimidine RNA precursor, so there was almost no RNA synthesis or growth before the addition of ^{14}C-uridine. The low incorporation of *ML210** (**Table 3**) may due to a low RNA synthesis rate or cell growth rate under starvation. Moreover, ppGpp has positive effects on

mRNA transcription (Stephens *et al*. 1975) which may consume a portion of ^{14}C-uridine, and exchange between the incorporated ^{14}C-uridine and the unlabeled exogenous uridine (Eduardo *et al*. 1976) may also interfere in the result of incorporation experiments.

Some ideas for future studies

ppGpp may perform similarly as GTP in activation of uracil phosphoribosyltransferase (UPRTase) (Jensen and Mygind 1996). UraA, which sits on the cell membrane and facilitates uracil entrance into the cytoplasm (Andersen *et al*. 1995), may be the restraining factor in uracil uptake. ppGpp may regulate the transcription of *uraA* gene or have a direct inhibitory effect on UraA. Based on these assumptions, other workers can carry out *vitro* experiments between UraA, *uraA* operon expression and ppGpp to obtain further information about uracil uptake system!

Materials and methods

Media and Strains

1. Media

Luria–Bertani broth (LB) contained 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per L deionized water, adjusted to pH 7.0 with 5 N NaOH, and 5 ml 1 M $CaCl_2$ and 10 ml 20% glucose were added after autoclaving for 20 minutes at 15 psi on liquid cycle. To make plates (LA), 15 g agar (Difco) was added per L LB. Kanamycin (kan, 15 µg/ml) and tetracycline (tet, 15 µg/ml) were added as needed. To make soft agar, 6 g agar (Difco) was added per L LB. (Miller 1991). Bochner plates (LA+FA) contained fusaric acid (12 µg/ml), chlortetracycline hydrochloride (50 µg/ml), $NaH_2PO_4 \cdot H_2O$ (10 mg/ml) and zinc chloride (0.1mM) in LA (Bochner *et al*. 1980).

The minimal medium M9 contained 6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 1 g NH_4Cl per L deionized water. 1 ml 1 M $MgSO_4$, 100 µl 1 M $CaCl_2$, 10 ml 20% glucose and 100 µl 10 mg/ml thiamine (VB1) were added after autoclaving for 20 minutes at 15 psi on liquid cycle. To make plates (M9 agar) 15 g agar (Difco) was added per L M9. (Miller 1991) Supplements to M9 were added as needed at the concentration listed in **Table I**, unless otherwise noted.

Table I. M9 supplements.

Supplement	Concentration
Casamino acid (CAA)	0.2%, a hydrochloric acid hydrolysate of casein and supplies a completely hydrolyzed protein nitrogen source[a]
Tryptophan (trp)	L-tryptophan, 5 µg/ml
aa	L-leucine, L-lysine, L-histidine, L-valine, L-isoleucine, all 100 µg/ml
Uracil (ura)	10 µg/ml
Uridine (uri)	10 µg/ml
Valine (val)	L-valine, 100 µg/ml
Isoleucine (ile)	L-isoleucine, 100 µg/ml
SMG[b]	L-threonine, L-leucine, L-arginine, L-histidine, all 100 µg/ml; L-serine, L-methionine, L-glycine, all 150 µg/ml
SMG control[b]	L-threonine, L-leucine, L-arginine, L-histidine, all 100 µg/ml
smg	L-serine, L-methionine, L-glycine, all 150 µg/ml
^{14}C-uridine[c]	^{14}C-uridine (500 mCi/mmol) plus uridine (10 mg/ml),

[a] USB. 2006.

[b] Uzan and Danchin 1976.

[c] Amersham Phamacia Biotech.

2. Strains

The genotype details of all strains used or constructed in my study are shown in **Table II**.

Table II. All *E. coli* strains in my study

Strain	Genotype	Source
SØ1259	*araD139*, Δ(*lac)U169*, *pyrF*, *rpsL*, *thi*	Laboratory collection
ML100	*araD139*, Δ(*lac*)*U169*, *pyrF*, *rpsL*, *thi*, *valS*, *zjh*::Tn*10*	This work
ML101	*araD139*, Δ(*lac*)*U169*, *rpsL*, *thi*, *valS*, *zjh*::Tn*10*	This work
ML102	*araD139*, Δ(*lac*)*U169*, *pyrF*, *rpsL*, *thi*, *valS*, *uraA*::Tn*10*	This work
ML110	*araD139*, Δ(*lac*)*U169*, *relA2*, *pyrF*, *rpsL*, *thi*, *zga*::Tn*10kan*	This work
CP78	*thr1*, *leuB6*, *his65*, *argH46*, *thi1*, *ara13*, *gal3*, *malA1*, (λ^r) *xyl7*, *mtl2*, *tonA2*, *supE44* [a]	Laboratory collection
CP79	*thr1*, *leuB6*, *his65*, *relA2*, *argH46*, *thi1*, *ara13*, *gal3*, *malA1*, (λ^r) *xyl7*, *mtl2*, *tonA2*, *supE44* [a]	Laboratory collection
SØ1345	*lacZ*, *rpsL*, *thi*, *uraA*::Tn*10*, *pyrF30* [b]	Laboratory collection
RH491	*leu*, *lac*, *trp*, *his*, *ilv*, *lys*, *rpsL*	Laboratory collection
ML200	*leu*, *lac*, *pyrF*, *trp*, *his*, *ilv*, *lys*, *rpsL*, *valS*, *zjh*::Tn*10*	Laboratory collection
ML201	*leu*, *lac*, *trp*, *his*, *ilv*, *lys*, *rpsL*, *valS*, *zjh*::Tn*10*	This work
*ML210**	*leu*, *lac*, *pyrF*, *trp*, *his*, *ilv*, *relA2*, *lys*, *rpsL*, *valS*, *zga*::Tn*10kan*	This work

[a] Magnusson *et al*. 2005.

[b] Andersen *et al*. 1995.

Growth of P1

1 ml overnight LB culture of *E. coli* was mixed with 0.1 ml $CaCl_2$, 0.2 ml 20% glucose, 50 μl P1 lysate given by Robert Fast and 4 ml soft agar (around 45℃), and poured out onto an LA plate. The plates were incubated overnight. The next day, 5 ml LB was added to each plate to harvest the upper soft agar from the LA plate. After 45 minutes at room temperature, this LB-soft agar mixture was centrifuged at about 3400×g in bench centrifuge (Wifug Lab) at 4℃ for 30 minutes. The supernatant was filtered through a 0.45 μm filter (Sarstedt Germany). The lysate was stored at 4℃ (Hughes *et al*. 2006). All the P1 lysates details are shown in **Table III**.

Table III. P1 lysate prepared and used in this project

P1 lysate	Host Strain	Medium	Incubation Temp ℃
pyrF$^+$	*MG1655*	LA	37
valS zjh::Tn*10*	*UT153*	LA+tet	30
zga::Tn*10kan*	*MG1655*	LA+kan	37
relA2 zga::Tn*10kan*	*CP79* relA$^-$ Kan^R	LA+kan, SMG, SMG Control	37
uraA::Tn*10*	*SØ1345*	LA+tet	37

Strains Construction

1 ml LB overnight culture of *E. coli* (recipient strain) was transferred into a sterile microfuge tube and the cells were spun down in a microcentrifuge (Biofuge Pico), 9500×g for 2 minutes. The cell pellet was resuspended in 0.5 ml 10 mM $CaCl_2$ 5 mM $MgSO_4$ by vortexing. 100 µl of this cells suspension was mixed gently with 10, 25 or 50 µl P1 lysate. The mixes were incubated 20 minutes at 37℃ in a water bath to permit the phage to absorb to the cells. After that, 200 µl Na-citrate and 1 ml LB were added to prevent further infection by phage. The cells were spun down at 9500×g and resuspended in 50 µl LB. The bacteria were spread on a selective agar plate. As a control, 100 µl of the original bacterial culture without any phage was plated out on the same plate. Next day, transductants were picked and purified on the same selective agar plate.

The *relA* transductant was first selected on the LA+kan plate, then the Kan^R colony was picked and screened by Uzan's SMG test (Uzan and Danchin 1976): the Kan^R transductant was streaked both on a plate supplemented with L-serine, L-methionine and L-glycine (SMG or M9+ura+smg) and a control plate (SMG control or M9+ura). A *relA* mutant grew well on the control plate but not the SMG plate, while the $relA^+$ strain grew well on both of them. The selection medium and incubation temperature of each mutant is shown in **Table IV**.

Table IV. Material details in constructions

Mutant	Recipient Strain	P1 lysate	Selective and screening medium	Temp ℃
ML100	*SØ1259*	*valS zjh*::Tn*10*	LA+tet	30
ML110	*SØ1259*	*relA2 zga*::Tn*10kan*	LA+kan; M9+ura+smg, M9+ura	37
ML101	*ML100*	$pyrF^+$	M9+CAA	30
ML102	*ML100*	*uraA*::Tn*10*	LA+FA; LA+tet	30
ML201	*ML200*	$pyrF^+$	M9+CAA+trp	30
*ML210**	*ML200*	*relA2 zga*::Tn*10kan*	LA+kan; M9+ura+CAA+trp+smg, M9+CAA+trp+ura; M9+ura+aa+trp+smg, M9+aa+trp+ura	30

Radioactive incorporation experiments

Direct amino acid starvation was achieved by incubation in minimal medium. 10^7 cells (100 µl) of *ML210** (or *ML200*) with 0.9 ml M9+val+ile or M9+aa+trp were incubated at 30℃. After 45 minutes incubation, 50 µl ^{14}C-uridine (50 mCi/mmol) was added into each culture. After 30 minutes incubation with ^{14}C-uridine, the culture was dropped into 5 ml ice-cold 5% trichloroacetic acid (TCA) and treated as described below. As a control 10^7 cells (100 µl) of *CP78* (or *CP79*) were treated the same way with M9+aa or M9+CAA at 37℃, and 100 µl ^{14}C-uridine (10 mCi/mmol) was added.

Indirect protein starvation was achieved by incubating $valS^{ts}$ strains at a restrictive temperature. 10^8 cells (1 ml) of *ML210** (or *ML200*) were incubated at 30℃ in 8 ml

M9+aa+trp. After 45 minutes (or 30 minutes) incubation, 140 µl (or 300 µl) ^{14}C-uridine (10 mCi/mmol) was added to the culture. Before the isotope was added, 1 ml culture was taken as a zero point. After the isotope was added, 1 ml sample was taken every 30 minutes (or 15 minutes). The sample was dropped into 5 ml ice-cold 5% TCA. The incubation temperature was shifted to 37℃ at 60 minutes after the zero point sample taken out (or 42℃ at 30 minutes).

TCA-precipitable radioactivity of each sample was estimated by the following procedure: 1 ml aliquots of bacterial culture were poured into 5 ml ice-cold 5% trichloroacetic acid (TCA) in a plastic tube where the cells were left for 30 minutes. The mixture of cells and TCA was filtered through a glass-fiber disk (Whatman, 3MM, GF/C), and the plastic tube that was used to carry the mix was washed three times with 5 ml TCA each (totally 15 ml) that were also filtered through the same disk. Last, the filter disk was treated by 95% ethanol once to remove the remnant TCA. The filter disc was dried at 40℃ for 1 hour and the amount of radioactivity on the disc was counted in a toluene scintillation mixture by the "Philip" liquid scintillation counter.

Growth experiments in different media or at different temperatures

E. coli was incubated in a LB or M9 for an overnight culture. Then, 1 ml overnight culture was transferred into 25 ml fresh medium. These cells were incubated by shaking at a certain temperature for 1 hour. When OD_{600} reached around 0.05, 1 ml sample was taken out at every 20 or 30 minutes and its OD_{600} (or OD_{450}) was measured in a spectrophotometer. The measurements would be stopped when OD_{600} reached 0.5 ~ 0.6, and a growth curve was plotted out on the semi-logarithmic paper (Hughes *et al*. 2006). 2×10^5 cells of *ML200* (or *ML201*) were transferred into 5 ml M9+CAA+trp+ura (or M9+CAA+trp) and incubated overnight in tubes at different temperatures: 30, 33, 36, 39, 42℃. The OD_{450} value was measured next day.

10 µl overnight culture of the putative *relA* mutant *ML210** were transferred into 3 ml M9+aa+trp with a certain uracil concentration in tubes and incubated by shaking (140/min) at 30℃ or 33.5℃ overnight. The OD_{600} of the bacteria culture was measured next day (18 h) and plotted as a function of uracil concentrations. As a control, *ML200* was used.

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