5-Fluoroorotate-resistant mutants of *Salmonella typhimurium*

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Spontaneously occurring mutants of *Salmonella typhimurium* resistant to 5-fluoroorotate (5-FOA) were isolated. One class of mutant showed marked derepression of pyrimidine biosynthetic enzymes and had the unusual property of being unable to grow on nutrient agar. However, when the osmotic strength of nutrient agar was increased, the mutants were able to grow. The genetic basis for the osmotic fragility and elevated *pyr* enzyme synthesis was the result of mutations affecting *pyrH*, encoding the enzyme uridine 5'-monophosphate kinase.

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On a isolé des mutants d'apparition spontanée de *Salmonella typhimurium* résistant au 5-fluoroorotate (5-ROA). Un type de mutant présente une dérépression marquée des enzymes biosynthétiques de la pyrimidine; de plus, il a la propriété exceptionnelle de ne pouvoir croître sur la gélose nutritive. Cependant, lorsque la pression osmotique de la gélose nutritive est augmentée, les mutants peuvent croître. On a démontré que le fondement génétique de cette fragilité osmotique et de la synthèse élevée de l'enzyme *pyr* est dû aux mutations affectant *pyrH*, codant l'enzyme uridine 5'-monophosphate kinase.

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Introduction

The *de novo* biosynthesis of the pyrimidine nucleotide, uridine 5'-monophosphate (UMP), in *Escherichia coli* and *Salmonella typhimurium* is accomplished by the sequential action of six enzymes encoded by six unlinked genes. In both of these genera, a single carbamyl phosphate synthetase (CPSase; encoded by *pyrA*) catalyzes the synthesis of carbamyl phosphate which is an intermediate in the biosynthesis of both pyrimidines and arginine (Fig. 1).

The patterns of genetic regulation of the *pyr* regulon are of interest since the structural genes for the *de novo* biosynthetic enzymes are individually scattered on the chromosome of *E. coli* (Bachmann *et al.* 1976) and of *S. typhimurium* (Sanderson 1972). Moreover, the expression of the nonlinked *pyr* genes, *pyrA–F*, is not regulated by a single corepressor. Both a cytidine nucleotide and arginine are involved in regulating the expression of *pyrA* (Abd-El-Al and Ingraham 1969). Recently it was shown that the *argR* product controls the synthesis of CPSase (Abdelal *et al.* 1976; Pierard *et al.* 1972); mutations in *argR* reduce the repressibility
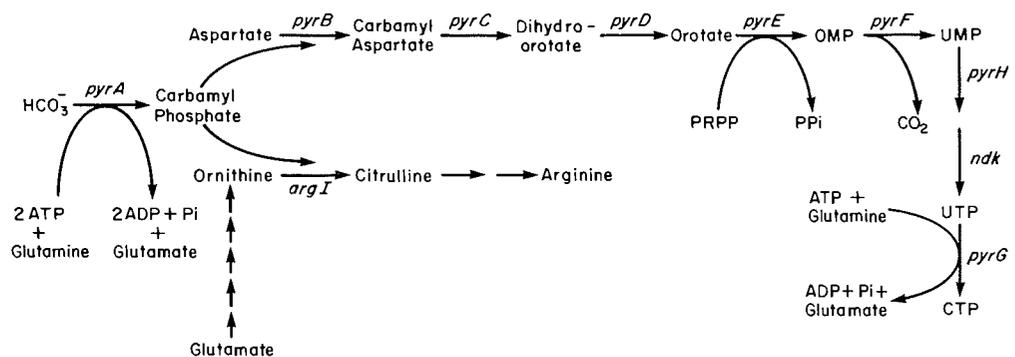

FIG. 1. Pyrimidine and arginine biosynthetic pathways of *Salmonella typhimurium*. Genetic symbols for the genes encoding the enzymes are shown in italics. The genetic designations are as follows: *pyrA*, carbamyl phosphate synthetase (CPSase, EC 2.7.2.5); *pyrB*, aspartate transcarbamylase (ATCase, EC 2.1.3.2); *pyrC*, dihydroorotase (DHOase, EC 3.5.2.3); *pyrD*, dihydroorotate dehydrogenase (DHOdehase, EC 1.3.3.1); *pyrE*, orotate phosphoribosyl transferase (OMPppase, EC 2.4.2.10); *pyrF*, orotidine 5'-monophosphate decarboxylase (OMPdecase, EC 4.1.1.23); *pyrH*, uridine 5'-monophosphate kinase (UMP kinase, EC 2.7.4.4); *ndk*, nucleoside-diphosphokinase (EC 2.7.4.6); *pyrG*, cytidine 5'-triphosphate synthetase (CTP synthetase, EC 6.3.4.2); and *argI*, ornithine transcarbamylase (OTCase, EC 2.1.3.3).

by both arginine and pyrimidines whereas the regulation of synthesis of the other pyrimidine biosynthetic enzymes remains unaffected (Kelln and O'Donovan 1976). Two independent studies (Kelln, Kinahan, *et al.* 1975; Schwartz and Neuhard 1975) have shown that the expression of *pyrB*, *pyrE*, and *pyrF* is controlled by a uridine nucleotide and that the expression of *pyrC* and *pyrD* is regulated by a cytidine nucleotide. The exact biochemical nature of the corepressors has not been resolved.

From the accumulated evidence, it is reasonable to assume that regulatory loci governing the expression of the *pyr* genes, *pyrB* through *pyrF*, do exist. To date, however, true regulatory mutants altered in the expression of these *pyr* genes have not been isolated. Mutants which mimic constitutive synthesis of all six pyrimidine biosynthetic enzymes have been isolated. These mutants do not have a regulatory dysfunction per se, but rather contain a partial impairment of the enzyme UMP kinase, encoded by *pyrH* (Ingraham and Neuhard 1972; Justesen and Neuhard 1975; Legrain *et al.* 1976; Fig. 1). The pyrimidine regulatory (*pyrR*) mutants reported previously (O'Donovan and Gerhart 1972) have now been verified as being *pyrH* (Justesen and Neuhard 1975). The *pyrH* allele is unlinked to any of the *pyr* structural genes encoding the biosynthetic enzymes (Bachmann *et al.* 1976; Sanderson 1972).

The use of pyrimidine analogues has proven valuable in studying various aspects of pyrimidine metabolism, but attempts to isolate pyrimidine regulatory mutants using pyrimidine analogues have not been successful (Justesen and Neuhard 1975). To our knowledge, 5-fluoro-analogues of metabolic intermediates of the pathway have not been used as a means for isolating pyrimidine regulatory mutants. Using different analogues for the isolation of regulatory mutants is not without precedence (Gots and Benson 1974). Thus, experiments were initiated to isolate and characterize mutants of *S. typhimurium* which were resistant to 5-fluoroorotate (5-FOA), the primary objective being the selection of true pyrimidine regulatory mutants.

Materials and Methods

Organisms
All bacterial strains used in this study were derivatives of *S. typhimurium* LT2 (Table 1). Strain JL1193 was the parent strain of all 5-FOA-resistant mutants isolated in this study. Bacteriophages P22 and F0 were from K. E. Sanderson and bacteriophage P1-kc was obtained from J. L. Ingraham.

Media
The minimal medium (TF) has been described by Edlin and Maaløe (1966); 0.3% glycerol (TFY medium) or 0.2% glucose (TFG medium) served as carbon sources. Nutritional supplements, when required, were added at a final concentration of 100 μg/ml. Complex media were Difco nutrient broth (NB) or LC broth (Kelln, Foltermann, and O'Donovan 1975). Solid media were prepared by addition of 15 g agar/l.

Growth Conditions
The temperature of incubation was 30°C unless stated otherwise. Liquid cultures were grown in sidearm flasks in a model G76 water bath shaker (New Brunswick Scientific Co., Inc.). Growth was followed by measuring the increase in absorbance at 660 nm.

Isolation of 5-FOA Resistant Mutants
Salmonella typhimurium is permeable to the pyrimidine intermediate orotate providing glycerol is the carbon source. Therefore, 5-FOA was added to TFY solid medium at various concentrations ranging from 5 μg 5-FOA/ml to 50 μg 5-FOA/ml. Strain JL 1193 was grown to stationary phase in TFY and 0.1-ml samples spread onto the selective media. After appropriate incubation (48–72 h), developing colonies were picked and purified.

Preparation of Cell Extracts and Enzyme Assays
The methods for preparing cell extracts and determining protein concentration have been reported (Kelln, Kinahan, *et al.* 1975). All enzyme assays were carried out at 37°C. Specific activities of enzymes were determined in conditions in which the rate of the reaction was proportional to extract concentration and time, and are expressed as nanomoles of product formed (or substrate utilized) per minute per milligram protein.

Genetic Techniques
The procedures for isolating Gal⁻ (genotype, *galE*) strains of *S. typhimurium* and for carrying out P1-kc mediated transductions with Gal⁻ recipients have been described (Kelln, Foltermann, and O'Donovan 1975).

Syntrophic Studies
For the detection of bacteria which overproduced pyrimidines, indicator plates were prepared as described by Kelln, Foltermann, and O'Donovan (1975) using strain F146 as the indicator bacterium.

Chemicals
Pyrimidines, amino acids, and various reagents for enzyme assays were obtained from Sigma Chemical Co. The 5-FOA was a generous gift from W. E. Scott, Hoffmann-La Roche Inc., Nutley, NJ.

Radiochemicals
The substrate, [4-¹⁴C] uridine 5'-monophosphate, was purchased from Amersham Corp.

Results

Phenotypic Properties of 5-FOA Resistant Strains
The parent strain JL1193 is temperature-sensitive for pyrimidine biosynthesis because of a mutation in *pyrC* which renders the strain auxotrophic for orotate at 42°C. All 5-FOA-resistant strains were tested for the ability to utilize orotate at 42°C; those isolates having acquired resistance to 5-FOA through mutations restricting the entry of orotate into the cell should fail to grow at this temperature. In total, sixty-three 5-FOA-resistant

TABLE 1. Bacterial strains

Strain	Genotype	Source (reference)
JL1193	pyrC1502 cdd-9 cod-8 tpp-1 udp-11	J. L. Ingraham (Beck et al. 1972)
JL1269	pyrH1609 cdd-7	J. L. Ingraham (Ingraham and Neuhard 1972)
F146	pyrF146	K. E. Sanderson
KR12	pan-3 hisD galE123	Own collection

TABLE 2. Phenotypic properties of 5-FOA-resistant mutants

Strain[a]	Generation time,[b] h	Pyrimidine excretion[c]	Growth condition[d]			
			NA	LC	FOA	FU
JL1193	1.8	−	+	+	−	−
JL1269	2.1	+	+	+	(+)	+
FOR1-10	3.0	+	−	+	+	+
FOR21-10	2.6	+	(+)	+	+	+
FOR39-20	2.9	+	−	+	+	+
FOR44-25	4.3	+	−	+	+	+
FOR63-50	3.3	+	−	+	+	+

[a]The first number following the three-letter designation FOR (an acronym for 5-fluoro-orotate resistant) is the number assigned to that particular mutant. The number following the hyphen denotes the concentration of 5-FOA (in µg/ml) used in the selective medium.
[b]Measured as the time required for the culture to double in absorbance at 660 nm.
[c]Excretion of pyrimidines was detected using strain F146 indicator plates. Symbols are: +, growth of indicator bacterium after 48 h incubation; −, no growth of indicator bacterium after 48 h incubation.
[d]The 5-FOA was added to TFY solid medium at a final concentration of 50 µg/ml. The 5-fluorouracil was added at a final concentration of 4 µg/ml to TFG solid medium (FU medium). Observations were made after 48 h incubation. Symbols are: +, growth; (+), weak growth; −, no growth.

strains were isolated and all isolates retained the ability to utilize orotate at 42°C.

For storage purposes, the isolates were transferred to nutrient agar (NA) stabs. Certain isolates did not grow (or grew very poorly) in this medium and these strains were chosen for further study. The properties of these nutrient agar negative strains (phenotypically referred to as NA⁻ strains), along with the parent strain and a known pyrH mutant (JL1269), are summarized in Table 2. All of the NA⁻ strains grew in the presence of 50 µg 5-FOA/ml whereas growth of the parent strain was inhibited and strain JL1269 grew poorly at this concentration of the analogue. Resistance to 5-FOA also conferred resistance to 4 µg 5-FU/ml.

Overproduction of pyrimidines (or intermediates of the pyrimidine pathway) would be an expected property of constitutive mutants producing high levels of pyrimidine biosynthetic enzymes. Since strain F146 is blocked at the last step of the pathway, all of the NA⁻ strains (and strain JL1269) excrete pyrimidines.

The growth rates of the NA⁻ strains were decreased dramatically from that of the parent strain. This suggested that the mutation conferring resistance to 5-FOA imparted an impairment in an essential metabolic process.

Osmotic Sensitivity Studies

Though the NA⁻ strains failed to grow on NA, they would grow on LC agar (Table 2). Further tests were conducted in order to determine the "growth factor" present in LC medium but presumably lacking in NA. The various chemically defined components of LC medium were added individually to NA at the same concentration used in preparing the LC medium. These media were then tested for the ability to support growth of the NA⁻ strains (Table 3). Addition of $CaCl_2$ or glucose at the defined amounts did not support growth whereas NA supplemented with NaCl did support growth.

NA plates containing $(NH_4)_2SO_4$ or lactose (both added at a concentration producing an equal osmotic strength to that of NaCl-supplemented plates) were prepared. The NA⁻ strains grew on both of these media (Table 3).

Levels of Pyrimidine Biosynthetic Enzymes

An expected phenotype for regulatory mutants would be an alteration in the rate of synthesis of various enzymes for a given pathway. The levels of four pyrimidine biosynthetic enzymes were measured in cells grown in repressing (uridine added) and derepressing conditions (uridine omitted). The

TABLE 3. Growth response of NA⁻ strains on supplemented NA media

Strain	Additions to NA medium[a]				
	NaCl	CaCl₂	Glucose	(NH₄)₂SO₄	Lactose
FOR1-10	+	−	−	+	+
FOR21-10	+	−	−	+	+
FOR39-20	+	−	−	+	+
FOR44-25	+	−	−	+	+
FOR63-50	+	−	−	+	+

[a]Supplements (g/ℓ) to NA were as follows: NaCl, 5.0; CaCl₂, 0.11; glucose, 1.0; (NH₄)₂SO₄, 7.5; lactose, 58.5. The observations were made after 24 h incubation. Symbols are: +, growth; −, no growth.

TABLE 4. Levels of *pyr* enzymes[a]

Strain	Uridine	ATCase	Sp.act.[b]		
			DHOdehase	OMPppase	OMPdecase
JL1193	+	4.8	6.9	21	16
	−	7.3	9.9	38	20
JL1269	+	310	35	190	40
	−	360	39	220	41
FOR1-10	+	820	30	250	74
	−	680	22	220	80
FOR21-10	+	780	17	190	42
	−	740	20	200	44
FOR39-20	+	870	34	220	59
	−	900	34	240	57
FOR44-25	+	1800	33	280	71
	−	1600	31	260	72
FOR63-50	+	1300	25	230	44
	−	1100	24	220	45

[a]The strains were grown in TFG medium and where indicated, uridine was added at a final concentration of 100 μg/ml.
[b]Specific activity is defined in the text. Enzyme abbreviations are as given in the legend to Fig. 1.

results are presented in Table 4. With strain JL1193, characteristic repression of the enzymes was observed when uridine was added to the medium. Strain JL1269 (*pyrH 1609*) had markedly elevated enzyme levels and only slight repression occurred when uridine was present in the medium. The NA⁻ strains exhibited marked derepression of the *pyr* enzymes, particularly with respect to ATCase; the ATCase level in all NA⁻ strains exceeded that observed for strain JL1269. No repression occurred when the NA⁻ strains were grown in the presence of uridine.

Genetics

The *pyr* enzymes in NA⁻ strains were derepressed and the levels remained unchanged when the strains were grown in standard repressing conditions, a pattern also observed with the *pyrH* mutant, strain JL1269. The *pyrH* gene has been located on the *S. typhimurium* chromosome at 7 min and is 30% cotransducible with *pan* when phage P1-kc is used as the transductional vector (Ingraham and Neuhard 1972). Experiments were carried out in

order to determine whether the 5-FOA-resistant character of NA⁻ strains was cotransducible with *pan*. The results of the transductional analyses are given in Table 5. For those strains examined, 5-FOA resistance was linked to *pan* at a frequency similar to that observed for the *pyrH 1609* allele. Difficulty was encountered in isolating Gal⁻ strains of some of the NA⁻ strains and therefore no genetic analyses were carried out.

UMP Kinase Activities of NA⁻ Strains

Genetic evidence indicated that the NA⁻ strains were *pyrH* mutants and the activity of UMP kinase in the individual strains was measured in order to substantiate or refute this hypothesis. The data presented in Table 6 clearly show that NA⁻ strains have reduced UMP kinase activity; in fact, the activity in some strains was virtually immeasureable. The argument could be made, however, that elevated *pyr* enzyme levels interfere with the assay of UMP kinase. Therefore, as a control, an equal volume of extract of strains JL1193 and strain FOR44-25 were mixed, and the UMP kinase ac-

TABLE 5. Transductional mapping of the mutation(s) conferring resistance to 5-FOA

		Transductants			
Donor[a]	Recipient	Selected marker	Unselected marker[b]	Unselected/ selected	% cotransduction
JL1269G	KR12	pan^+	$pyrH1609$	58/249	23.3
FOR21-10G	KR12	pan^+	$pyrX21$	37/129	28.7
FOR44-25G	KR12	pan^+	$pyrX44$	85/246	26.4

[a]Donor strains are Gal⁻ (galE) derivatives and are so indicated by the letter "G" which follows the original strain designation.
[b]The symbol pyrX is an arbitrary genotype assigned for mutation to 5-FOA resistance. The transfer of pyrX was initially assessed by screening the transductants for resistance to 5-FU (final concentration of 4 µg/ml in TFG medium).

TABLE 6. Level of UMP kinase activity of NA⁻ strains

Strain	Sp. act.
JL1193	27
JL1269	1.2
FOR1-10	0.21
FOR21-10	0.25
FOR39-20	0.28
FOR44-25	1.7
FOR63-50	2.0
JL1193 + FOR44-25[a]	25[b]

[a]The combined extract consisting of equal volumes of extract from strains JL1193 and FOR44-25 was assayed for UMP kinase activity.
[b]This value has been corrected for the dilution factor and the activity contributed by the FOR44-25 extract and thus represents the specific activity of the JL1193 extract in these conditions.

tivity determined. The addition of strain FOR44-25 extract did not influence the assay (Table 6).

Discussion

This study was initiated for the primary purpose of isolating pyrimidine regulatory mutants (genotypically pyrR) altered in the expression of pyr genes. Earlier attempts (O'Donovan and Gerhart 1972) to isolate such mutants by selecting strains which were resistant to the two pyrimidine analogues, 5-fluorouracil and 5-fluorouridine, were unsuccessful in that the isolates only mimicked pyrR mutants and have recently been characterized as mutants defective in the structural gene pyrH encoding UMP kinase (Justesen and Neuhard 1975).

Of the sixty-three 5-FOA-resistant strains isolated by us, several strains exhibited a rather novel phenotype; they failed to grow on NA but grew on synthetic medium and on LC medium. When various chemically defined components of LC medium were added to NA, the addition of NaCl alone would support growth. Other chemicals were substituted for NaCl and added at the same osmotic strength to NA. The NA⁻ strains grew, thereby showing that the remedial action of NaCl initially observed was not restricted to this chemical. Thus, it would appear that the NA⁻ strains were osmotically fragile.

Temperature-sensitive osmotic-remedial (TSOR) mutants have been widely observed in enteric bacteria. Many temperature-sensitive mutants will grow at the non-permissive temperature when the osmotic strength of the medium is increased. These include strains affected in aminoacyl transfer RNA synthetase, DNA synthesis, and envelope structure (Chatterjee et al. 1976). The NA⁻ strains described here are not temperature-sensitive mutants but are, nonetheless, osmotic-remedial mutants.

Rough mutants (heptose-deficient, chemotype Re) of S. typhimurium and E. coli lack certain proteins in their outer membrane which are present in the smooth strain (Chatterjee et al. 1976). It has been proposed that defective enzymes regain activity in media of high osmolarity due to correction of tertiary structure. However, it has been observed that rough mutants of S. typhimurium still make defective lipopolysaccharide in the presence of high salt concentrations (Chatterjee et al. 1976). Thus, it would appear that for these rough strains the NaCl does not restore the activity of the mutated gene product but rather, may act by a mechanism which stabilizes the outer membrane.

The NA⁻ strains described here are smooth strains as indicated by their retention of sensitivity to bacteriophage P22 (Wilkinson et al. 1972). Pyrimidines (as sugar nucleoside diphosphates) are involved in cell envelope biosynthesis and even though the NA⁻ strains have retained the smooth character, possibly a mutation affecting pyrimidine biosynthesis could subtly alter the cell envelope such that the cell is now osmotically fragile but yet retains the smooth character. Alternatively, in the NA⁻ strains, the activity of an essential enzyme is restored to an adequate level at high salt concentrations and thus growth occurs. If this is the case, then given the evidence stated below, the essential enzyme would be UMP kinase.

The levels of the pyr enzymes in NA⁻ strains

were markedly elevated. The synthesis of the enzymes encoded by *pyrC* and *D* is regulated by cytidine nucleotides whereas control of synthesis of the enzymes encoded by *pyrB*, *E*, and *F* is regulated by uridine nucleotides (Kelln, Kinahan, *et al.* 1975; Schwartz and Neuhard 1975). Since *pyr* enzyme synthesis is regulated by different corepressors, and most likely different repressor proteins, it is unlikely that a mutation in a single regulatory gene would evoke simultaneous derepression of the *pyr* enzymes.

The pattern of regulation for *pyr* enzyme synthesis in NA⁻ strains was similar to that observed for the *pyrH* mutant, strain JL1269. Genetic studies showed that the 5-FOA-resistant character (genotypically referred to as *pyrX*) of the NA⁻ strains was cotransducible with *pan* at a frequency similar to that observed for *pyrH*. The transductants were phenotypically NA⁻ and derepressed for *pyr* enzymes, thus indicating that NA⁻, *pyr* enzyme elevation, and 5-FOA resistance were determined by a mutation in a single genetic locus or closely grouped loci.

The UMP kinase activity of the NA⁻ strains was markedly lower than that of the parent strain; most of the NA⁻ strains had less activity than strain JL1269. Impairment of UMP kinase activity results in reduced synthesis of UDP which consequently should reduce the endogenous concentration of other pyrimidine nucleotides, notably UTP. It has been reported previously (Schwartz and Neuhard 1975) that the size of the UTP pool as well as the growth rate of any particular *pyrH* mutant is dependent on the *pyrH* mutant in question. The more severe the impairment of UMP kinase activity, the slower the growth rate, the smaller the UTP pool, and the greater the derepression of *pyr* enzymes. As expected, the NA⁻ strains with the slowest growth rate (namely strains FOR44-25 and FOR63-50) were the most derepressed for *pyr* enzymes. However, these strains had greater in vitro UMP kinase activity than strain JL1269 or the other NA⁻ strains. This apparent discrepancy may simply be a consequence of variable in vitro stability of UMP kinase from the different NA⁻ strains and therefore does not accurately reflect the in vivo activity. Generation time and endogenous UDP and UTP pool sizes are more accurate reflections of in vivo UMP kinase activity.

The accumulated evidence indicated that the NA⁻ strains harbor mutations affecting *pyrH*. The reduced UMP kinase activities observed could result from a mutation in the *pyrH* structural gene, or alternatively, the mutation affects the regulation of

expression of *pyrH* resulting in reduced levels of the gene product. Selecting mutants which are resistant to 5-FOA and screening such mutants for pyrimidine overproduction and derepression of *pyr* enzymes should provide a simple method for isolating *pyrH* mutants. This approach has recently be used and proven successful (R. Bujaczek, personal communication).

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