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A *Salmonella typhimurium* Mutant Dependent upon Carbamyl Aspartate for Resistance to 5-Fluorouracil Is Specifically Affected in Ubiquinone Biosynthesis

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The isolation and properties of a mutant dependent upon exogenous carbamyl aspartate for resistance to 5-fluorouracil are described. The mutant was deficient in the synthesis of ubiquinone and accumulated a quinone provisionally identified as the ubiquinone precursor 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone. The mutation resulted in an alteration in the regulation of synthesis of enzymes involved in de novo pyrimidine biosynthesis but did not establish a functional block in dihydroorotate dehydrogenase activity in vivo. Conditional resistance to 5-fluorouracil apparently occurred through an inhibition of the conversion of the analog to the nucleotide level.

Interest in pyrimidine nucleotide biosynthesis has originated primarily through the importance of these molecules as components of nucleic acids. However, the role of pyrimidine nucleotides in cellular physiology is not restricted to nucleic acid biosynthesis; pyrimidine nucleotides play crucial roles in carbohydrate metabolism, lipid metabolism (11), and lipopolysaccharide biosynthesis (10). Furthermore, factors controlling the utilization of pyrimidine nucleotides in various metabolic processes are unresolved, and the influence that such processes have on the overall regulation of pyrimidine enzyme synthesis is likewise unknown.

During the course of a study on pyrimidine metabolism and its regulation, a mutant was isolated which had the novel phenotype of dependency upon the pyrimidine intermediate carbamyl aspartate for resistance to 5-fluorouracil. The present report describes the isolation and characterization of this mutant and illustrates that the primary effect of the mutation relates to ubiquinone biosynthesis, which further affects several aspects of pyrimidine metabolism.

All bacterial strains used in this study are derivatives of *Salmonella typhimurium* LT2. Table 1 lists the numerical designations, genotypes, and source or method of derivation. The composition of the minimal medium, TFG medium (contains 0.2% glucose as a carbon source), and the complex medium, LC medium, has been described previously (16). Carbon sources other than glucose were added to the basal minimal salts at a final concentration of 0.3%. Nutritional supplements, when required, were added at 50 μg/ml.

Carbamyl aspartate-dependent, 5-fluorouracil-resistant mutants were isolated by plating strain B118-C onto TFG medium supplemented with 50 μg of carbamyl aspartate per ml and 10 μg of 5-fluorouracil per ml, followed by incubation at 37°C for 72 h. Resistant mutants were purified and further tested to identify unwanted isolates wherein resistance to 5-fluorouracil was a consequence of a mutation in the *upp* gene, encoding uracil phosphoribosyltransferase (EC 2.4.2.9). Strains lacking uracil phosphoribosyltransferase activity fail to convert uracil to UMP or 5-fluorouracil to 5-fluoro-UMP and consequently are resistant to the analog (2). Thus, *upp* mutants were recognized by their inability to use uracil as a pyrimidine source. Isolates which retained the ability to use uracil were transduced to pyrimidine prototrophy, and the transductants were screened for the property of carbamyl aspartate-dependent 5-fluorouracil resistance. A transductant (KR42) having this property was chosen for further study.

In either minimal medium or complex medium, the growth rate of KR42 was half that of KR2 (isogenic with KR42 except for the mutation under study). In addition, KR42 produced a low growth yield in cultures limited for glucose, attaining a final cell mass which was one-third that of strain KR2. Significantly, KR42 grew poorly with glycerol as a carbon source and did not utilize either citrate or succinate as a carbon source. From these observations, it was inferred that the strain was impaired in energy metabolism.

The position of the mutation imparting the pleiotropic phenotype of KR42 has been established at a point between units 14 and 15 (8a) on the current linkage map of *S. typhimurium* (12). No previously described gene mapping in this region of the *S. typhimurium* chromosome ap-

peared to be allelic with the mutation. However, located within the corresponding region of the *Escherichia coli* K-12 chromosome (1) is the *ubiF* gene (encoding a gene product necessary for ubiquinone biosynthesis). Since accumulated evidence (see discussion below on measuring the activity of dihydroorotate dehydrogenase) had indicated a deficiency in electron transport in KR42, we proceeded to investigate whether the mutant was ubiquinone deficient.

Chromatography of acetone-ligroin extracts from KR2 and KR42 with solvent A yielded similar chromatograms, with both having a single yellow-colored band of the same R_f value (Table 2). A yellow color is characteristic of ubiquinone and quinone intermediates of ubiquinone biosynthesis (5). In solvent B, the compound from KR42 migrated more slowly than that from KR2. UV absorption spectra were taken, and the λ_{max} for each compound was determined (Table 2). The compound isolated from KR2 gave a spectrum typical of ubiquinone (literature value for λ_{max} = 274 nm [5]). The spectrum of the compound from KR42 was characteristic of the ubiquinone intermediate 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone (literature value for λ_{max} = 266 nm [5]), which migrates more slowly than ubiquinone in solvent B and has been shown to accumulate in *ubiF* mutants of *E. coli* (5, 15). Thus, on the basis of both biochemical and genetic evidence, we infer that KR42 is a *ubiF* mutant (assuming that the genetic and biochemical organization for ubiquinone biosynthesis is the same in *S. typhimurium* and *E. coli*).

TABLE 1. *Bacterial strains*

Strain	Genotype[a]	Source or method[b]
B118-C	*pyrB118 usp-3*	Laboratory collection
KR1	*argI539 usp-3*	Laboratory collection
KR2	*usp-3*	Transduction of B118-C to *pyrB*$^+$
KR42	*usp-3 ubiF2*	Spontaneous mutant of B118-C, followed by transduction to *pyrB*$^+$

[a] The genetic symbol *usp* to indicate mutations producing permeability to ureidosuccinate (i.e., carbamyl aspartate) has been invoked previously (13) and has been approved by K. E. Sanderson of the Salmonella Genetic Stock Centre. Evidence for designating KR42 as a *ubiF* mutant is given in the text.

[b] Bacteriophage strain L4-2 (an *int-4 cly-2* double mutant of phage P22) was used for transductions (4) after propagation on the donor strain, KR1.

TABLE 2. *Properties of compounds from acetone-ligroin extracts of KR2 and KR42*[a]

Source of compound	R_f value[b]		λ_{max}[c] (nm)
	Solvent A	Solvent B	
KR2 extract	0.15	0.7	274
KR42 extract	0.15	0.6	267

[a] Except for minor modifications, the isolation and characterization of the quinones were carried out by procedures established previously (5, 15). Cells (6 to 9 g, wet weight) were continuously extracted with 200 ml of acetone for 3 h. After evaporation of the acetone extract to dryness, the residue was extracted with 50 ml of ligroin (bp, 60 to 80°C).

[b] The acetone-ligroin extracts were concentrated and then separately applied to a thin-layer silica gel plate for chromatography. Solvent A (chloroform-ligroin [70:30, vol/vol]) was used for the initial separation of quinone compounds, and after elution of the compounds with diethyl ether, chromatography in solvent B (ethyl acetate-hexane [25:75, vol/vol]) was used to achieve further purification.

[c] The isolated compounds (obtained after chromatography in solvent B) were eluted from the silica gel plates with diethyl ether, and UV spectra (in diethyl ether) were obtained with a Pye-Unicam SP1800B spectrophotometer.

The effect that the mutation had on pyrimidine enzyme activities was assessed (Table 3). Cell extracts of KR42 exhibited limited dihydroorotate dehydrogenase activity when assayed by a procedure which requires that oxygen serve as the terminal electron acceptor (14). A deficiency in ubiquinone biosynthesis is congruent with the low activity observed; during aerobic growth of *E. coli*, electrons from the oxidation of L-dihydroorotate are transferred to a system involving ubiquinone and functional cytochromes (9, 14), and presumably a similar situation occurs in *S. typhimurium*. With an alternative assay procedure which utilizes dichlorophenol-indophenol as an exogenous electron acceptor, significant dihydroorotate dehydrogenase activity was shown to be present in KR42 extracts.

Table 3 includes results regarding the levels of pyrimidine enzyme activities in cells grown under repressing and nonrepressing conditions. In the absence of uracil or carbamyl aspartate, pyrimidine enzyme levels in KR42 were, in general, twice those in KR42. Growth in the presence of uracil reduced the levels of activities in KR42 to virtually the same values as for KR2. The repressive effect of carbamyl aspartate in KR2 was, at best, equal to that of uracil, similar to the effect observed in *E. coli* with another pyrimidine intermediate, orotate (3). In KR42, repression conferred by carbamyl aspartate was novel, mediating a higher degree of repression than that conferred by uracil.

Several independent studies (cited in reference 8) have shown that the synthesis of corepressors for *pyr* genes occurs subsequent to the synthesis of UMP. In KR42, if the low in vitro dihydroorotate dehydrogenase activity (evident in the absence of an exogenous electron acceptor) accurately reflected the in vivo activity, the increased derepression of pyrimidine enzyme

synthesis may have resulted from a reduced rate of de novo UMP biosynthesis. However, the addition of carbamyl aspartate mediated a greater repression of pyrimidine enzyme synthesis than did uracil, indicating that the in vivo dihydroorotate dehydrogenase activity facilitated an increased rate of UMP synthesis and, ultimately, corepressor synthesis. The evidence supports the conclusion that a functional block in dihydroorotate dehydrogenase does not occur in the *ubiF* mutant and that, if any stage of de novo pyrimidine nucleotide biosynthesis is rate limiting, it is the synthesis of carbamyl aspartate.

Studies were initiated to determine the basis for the conditional resistance to 5-fluorouracil displayed by KR42. Uracil and 5-fluorouracil are metabolized by the same routes in *S. typhimurium* (2); thus, any effect of carbamyl aspartate on the metabolism of 5-fluorouracil should also be expressed in the metabolism of uracil. In KR42, the addition of carbamyl aspartate prevented the incorporation of uracil into acid-precipitable material, whereas KR2 was unaffected

TABLE 3. *Levels of pyrimidine enzymes in KR2 (ubiF$^+$) and KR42 (ubiF2)*

Strain	Addition[a]	Sp act[b]				
		ATCase	DHOase	DHOdehase[c]	OPTase	OMPdecase
KR2	None	4.8	100	7.5 (3.4)	22	11
	Uracil	3.4	83	5.1	17	9.5
	Carbamyl aspartate	3.0	93	6.1 (3.1)	18	10
KR42	None	13	200	0.7 (6.5)	43	17
	Uracil	3.1	110	0.6	16	6.9
	Carbamyl aspartate	1.7	90	0.8 (2.7)	8.8	4.7

[a] Strains were grown in TFG medium at 37°C and, where indicated, in the presence of 50 μg of uracil or carbamyl aspartate per ml.

[b] Enzyme assays were carried out as previously described (7, 8). Abbreviations: ATCase, aspartate transcarbamylase (EC 2.1.3.2); DHOase, dihydroorotatase (EC 3.5.2.3); DHOdehase, dihydroorotate dehydrogenase (EC 1.3.3.1); OPTase, orotate phosphoribosyltransferase (EC 2.4.2.10); OMPdecase, orotidine 5′-monophosphate decarboxylase (EC 4.1.1.23). Specific activities were determined at 30°C and represent nanomoles of substrate transformed per minute per milligram of extract protein under conditions in which product formation (or substrate disappearance) was proportional to the extract and time. Values represent the average of three independent determinations, except for values within parentheses, which represent the average of two independent determinations.

[c] Specific activities were obtained by an assay which relies on the endogenous electron-accepting capacity of the cell extract (14), except for those within parentheses, which were determined by a procedure in which dichlorophenol-indophenol serves as an exogenous electron acceptor (7).

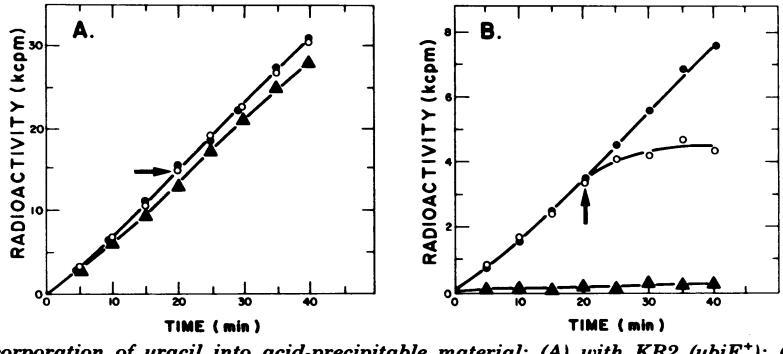

FIG. 1. *Incorporation of uracil into acid-precipitable material: (A) with KR2 (ubiF$^+$); (B) with KR42 (ubiF2). Strains were grown at 30°C to 2 × 10^8 cells per ml in TFG medium (●) or TFG medium containing 50 μg of carbamyl aspartate per ml (▲); (○) TFG medium with carbamyl aspartate added at 20 min (arrow) to a final concentration of 50 μg/ml. At zero time, [6-^{3}H]uracil (25 μM; 30 μCi/μmol) was added. Samples were transferred to equal volumes of 10% trichloroacetic acid and allowed to stand on ice for 20 min. Acid-precipitable material was then collected on Millipore membranes (0.45-μm pore diameter) which were prewashed with a cold solution of 1 mM uracil (unlabeled) dissolved in 5% trichloroacetic acid. The collected precipitate was washed twice with the 1 mM uracil–5% trichloroacetic acid solution and then dried. The amount of radioactivity in the precipitates was determined by scintillation counting.*

(Fig. 1). The effect in KR42 was rapid, with the incorporation of uracil ceasing within 5 to 10 min after the addition. This observation suggests that the effect does not result from an inhibition of synthesis of one or more enzymes necessary for the metabolism of uracil but more probably relates to events directly involved in the conversion of uracil to UMP.

KR42, a *ubiF* mutant, was isolated through conditional resistance to a pyrimidine analog although the isolation of a *ubiF* mutant was not specifically intended at the outset of the project. The situation is similar to the previously reported isolation of a leaky *guaB* mutant of *S. typhimurium* through resistance to a combination of pyrimidine analogs (6). In the *ubiF* mutant (KR42) and the *guaB* mutant, an alteration of *pyr* gene expression seems apparent. Whether the alteration results from a specific interrelationship of ubiquinone metabolism and *pyr* gene expression or stems from a more generalized physiological effect remains to be ascertained.

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