Journal of Industrial Microbiology, 5 (1990) 313–322
Elsevier

SIM 243

Elevated uridine nucleotide pools in fluorouracil/fluorouridine resistant mutants of *Nocardia lactamdurans*

Joyce Greene, Mark Rosenbach and Gary Darland

Merck Sharp and Dohme Research Laboratories, Rahway, NJ, U.S.A.

Received 16 May 1989
Revised 19 July 1989
Accepted 4 August 1989

Key words: Nocardia lactamdurans; Efrotomycin; Fluorouracil; UMP

SUMMARY

Selection of spontaneous mutants of *Nocardia lactamdurans* MA2908 for resistance to 5-fluorouracil results in the simultaneous development of resistance to 5-fluorouridine. The resulting mutants fall into four distinct classes based on the amount of uracil accumulating in fermentation broths. An additional characteristic of these mutants is a reduction in the ability to incorporate exogenous uracil into nucleic acids even though transport and conversion to the nucleotide level appears normal. Finally, production of efrotomycin is increased in these mutants in both chemically defined and complex fermentation media to levels equivalent to those of MA4820, the first productivity mutant isolated in a conventional strain improvement program. Resistance development and uracil excretion are adequately explained by an elevation of the intracellular uridine nucleotide pool, in particular UMP. The role of the uridine necleotides in the efrotomycin fermentation is unknown.

INTRODUCTION

Efrotomycin, a respresentative of the kirromycin class of antibiotics [18], was discovered in fermentation broths of *Nocardia lactamdurans*[1] [21]. It has shown efficacy in the treatment of swine dysentery [4] as well as potential as a growth permittant [3].

Initial efforts at strain improvement emphasized classical techniques of mutation and random screening for superior productivity. While this strategy was successful, and unexpected consequence is the tendency of suboptimal fermentations to accumulate uracil in the medium; concentrations in excess of 10 mM are not uncommon under these circumstances. This phenomenon was observed

[1] Orginally named *Streptomyces lactamdurans*, it was reclassified on the basis cell wall chemistry [22].

Correspondence: G. Darland, Merck Sharp and Dohme Research Laboratories, Rahway, NJ 07065, U.S.A.

with neither the original soil isolate nor strains of the cephamycin C lineage (Greene and Darland, unpublished). Furthermore, it was observed that the addition of uracil to production medium has an inhibitory effect on the efrotomycin fermentation (Darland, unpublished).

The following study was undertaken in an attempt to understand this phenomenon. The assumption was that uracil accumulation was detrimental and that by determining its origin and eliminating it, productivity would increase. The results in this paper and one by Nielsen [15] argue that with the isolation of the first superior culture (MA4820), a mutation was introduced which resulted in the derepression of the pyrimidine biosynthetic pathway. The net result is an increase in the intracellular UMP pool with resultant uracil excretion. Rather than being detrimental, as expected the mutation seems to be associated with increased productivity.

MATERIALS AND METHODS

Materials

[2-^{14}C]-uracil (57.4 mCi/mmol), [2-^{14}C]-uridine (50 mCi/mmol) and [2-^{3}H]-adenine (15Ci/mmol) were obtained from New England Nuclear. 5-Fluorouracil (FU) and 5-fluorouridine (FUr) were obtained from Sigma.

Bacteria

All the bacterial strains discussed below were derived from *N. lactamdurans* MA2908. This organism was previously identified as *Streptomyces lactamdurans* and deposited with the Northern Regional Research Laboratories (NRRL 3802). The bacteria were maintained as frozen vegetative mycelia (FVM) prepared in defined medium.

Media

The basal defined medium for the growth of bacteria consisted of (amount): NH_4Cl, 2 g; K_2HPO_4, 1.3 g; NaCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; aspartic acid, 0.5 g; monosodium glutamate, 8.25 g; sodium citrate, 0.5 g; inositol, 0.2 g; $CaCO_3$, 25 mg; $FeSO_4 \cdot 7H_2O$, 25 mg; $MnSO_4 \cdot 5H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 10 mg and $CuSO_4 \cdot 5H_2O$, 2 mg. The presterile pH was adjusted to 7.0. Poststerile additions of glucose and glycerol were made to a final concentration of 2% (w/v).

Efrotomycin production and uracil excretion were determined in the same medium modified by the elimination of glycerol and citrate and the inclusion of 3 g/l gelatin, 18.4 g/l soybean oil and 0.75 g/l hydroxypropylmethyl cellulose as an emulsifying agent.

The complex fermentation medium consisted of (g/l): Pharmamedia (Traders Protein), 58.0; soybean oil, 80; lactic acid, 2.0, lecithin, 2.5, polyglycol 2000 and 10 ml/l of a trace elements solution ($FeSO_4 \cdot 7H_2O$, 1 g/l; $ZnSO_4 \cdot 7H_2O$, 0.2 g/l; $MnSO_4 \cdot 5H_2O$, 1 g/l; $CuCl_2 \cdot 2H_2O$, 25 mg/l; $CaCl_2 \cdot 2H_2O$, 25 mg/l; H_3BO_3, 56 mg/l and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 19 mg/l in 0.6 N HCl).

ISP4 (Difco) was used as a routine plating medium and as a source of fragmented aerial mycelium.

Mutant isolation and confirmation

Spontaneous mutants resistant to 5-fluorouracil or 5-fluorouridine were selected by spreading approximately 10^7 cfu/plate on KN-1 agar supplemented with 1 mg/ml FU or 10 μg/ml FUr. After seven days at 28°C, colonies were reisolated under nonselective conditions and confirmed on appropriately supplemented media. KN-1 consists of (in grams per liter distilled water): NH_4Cl, 1.0; KNO_3, 2.0; K_2HPO_4, 2.0; NaCl, 0.5; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $ZnSO_4 \cdot 7H_2O$, 0.01 and $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 7.2 with NaOH and agar (20 g/l) was added. Post sterile addition of glucose was made to a final concentration of 1% (w/v).

Determinations of the minimum inhibitory concentration (MIC) and the isolation of resistant mutants were determined by spotting 10 μl containing 10^4 to 10^5 cfu on minimal medium (KN-1) supplemented with the appropriately diluted inhibitor.

Analytical procedures

Early to mid exponential phase cells were used in all pyrimidine incorporation studies. Cells were grown for 17–24 hours in defined medium at which

time the optical density at 600 nm ranged from approximately 3–10. These cells were diluted to an OD_{600} of 1.0 into fresh medium supplemented with 250 μM uracil, uridine or adenine as indicated. The cells were incubated for 45 min at 29°C at which time radioactive isotope was added to a final specific activity of 1 mC/mmol. Incorporation of isotope into TCA precipitable material was monitored over 2.5 h. During this interval less than 10% of the incorporated isotope was associated with NaOH stable material and therefore is assumed to represent RNA synthesis.

The determination of uracil and uridine transport was performed on cells that were similarly grown. The cells were washed in TKMC buffer (0.2 M tris, pH 8.0; 50 mM $MgCl_2$; 20 mM KCl and 20 mM $CaCl_2$) and resuspended in one third culture volume of TKMC. The washed cells were added to an equal volume of 0.4% glucose and preincubated for 5 min at 29°C before the addition of carrier free ^{14}C uracil to a final concentration of 25 μM. Incubation was performed in a water jacketed beaker and the cells were kept suspended by means of a magnetically driven stirbar. Uptake was monitored over the next four minutes by filtration of 0.2 ml aliquots and rapidly washing with 10 ml of 0.5× TKMC.

To determine the distribution of radioactivity within the acid soluble pool, the entire reaction mixture (3.0 ml) was filtered and rapidly washed with 20 ml 0.5× TKMC supplemented with 2.5 mM each of uracil and uridine. The filters were extracted with 2 ml ice cold 1 M formic acid. A 1.5 ml aliquot was dried under vacuum and redissolved in 50 μl water containing 150 nmol each UMP, UDP and UTP. After chromatography on PEI-cellulose with 1 M LiCl, the TLC plate was dried and counted with a System 200 Imaging Scanner (Bioscan, Inc.).

The cellular nucleotide pools were measured by filtration of 20 ml of a 24-h culture of bacteria and washing with 5 ml of water. The filter was placed in 10 ml of ice cold 1 M formic acid, the entire operation took about 90 s. The extracts were kept on ice for 1–2 h, centrifuged to remove cellular debris, filtered through 0.22 m Nylon filters (Corning) and lyophilized. The lyophilized extract was redissolved in water and analyzed by ion pair chromatography essentially as described by Payne and Ames Quantitation utilized external standards of known concentration. Peak identification was confirmed by co-chromatography with known compounds.

Efrotomycin (FR02) concentrations were determined by HPLC on PRP-1 using an isocratic solvent system, 60% solvent A (KH_2PO_4, 1.36 g/l; 1.83 g/l Na_2HPO_4) and 40% acetronitrile. Uracil concentration was determined on a Zorbax ODS column with 40 mM NH_4 acetate: methanol (99:1).

Protein concentration was determined on TCA precipitated material with the bicinchoninic acid reagent by using the enhanced (60°C) protocol described in the manufacturer's (Pierce Chemical) brochure.

RESULTS

Incorporation of uracil into TCA precipitable material

This work was undertaken to determine the cause of uracil excretion. Early experiments of the type indicated in Fig. 1 suggested that random mutagenesis of the original soil isolate, MA2908, and screening for improved productivity had inadvertently resulted in a significant reduction in the ability to incorporate exogenous uracil into TCA precipitable material. Adenine incorporation is also reduced but the effect is less dramatic. (Fig. 1). The phenotype appeared in the first improved mutant (MA4820)

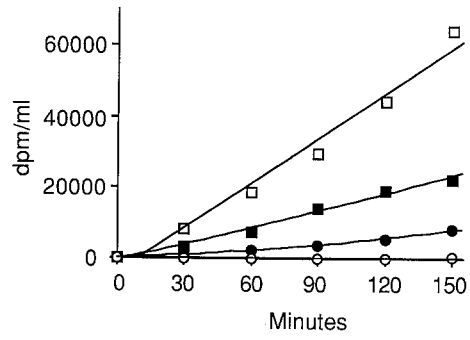

Fig. 1. Simultaneous incorporation of [2-^{14}C]-uracil and [2-^{3}H]-adenine into TCA precipitable material by *N. lactamdurans*. Squares, MA2908; circles, MA4820. Filled in symbols, adenine; open symbols, uracil.

and remains in isolates derived from this strain. The defect appears unrelated to growth rate since the generation times of MA2908 and MA4820 in defined medium are virtually identical.

With the discovery of this phenomenon, the efrotomycin lineage of *N. lactamdurans* was examined for susceptibility to a variety of pyrimidine analogs. It was determined that the original isolate, MA2908, differed from all derivatives with regard to susceptibility to both 5-fluorouracil and 5-fluorouridine. MA2908 was susceptible to 10 μg/ml of both agents, while MA4820 and later mutants were resistant to concentrations greater than 1 mg/ml (data not shown).

Isolation and characterization of 5-FU and 5-FUr resistant mutants

Armed with the above information it was decided to apply 5-FU and 5-FUr selection to MA2908. Twenty spontaneous mutants (Table 1) were isolated. After confirming that the mutations were stable, the mutants were grown in defined production medium and uracil excretion determined by TLC analysis of 3 day fermentation broths. The FU resistant (FU^R) mutants were initially classified into four groups based on the intensity of the uracil spot. This semi-quantitative classification was later confirmed by HPLC analysis of fermentation broths. All mutants selected on FU simultaneously acquired resistance to fluorouridine.

Although it was anticipated that uracil excretion might accompany acquisition of resistance to 5-FU [16], the simultaneous increase in efrotomycin productivity was unexpected. Class I, non-excreting, mutants exhibit about a 50% increase in titer in defined production medium and about 30% in complex medium. An additional incremental increase (about 25%) is seen for classes II though IV. The apparent reduction in titer seen in defined medium for the class IV mutants probably reflects a reduction in biomass. The class IV mutants appear quite similar to MA4820 with regard to both efrotomycin production and uracil excretion. (Table 1).

Selection on FUr yielded mutants (FUr^R) which retained susceptibility to fluorouracil. These mutants neither excreted uracil nor showed any evidence of increased productivity.

Uracil incorporation into nucleic acids by resistant mutants.

A representative mutant from each class was selected for evaluation of the pyrimidine salvage

Table 1

Characterization of 5-fluorouracil and 5-fluorouridine resistant mutants of *N. lactamdurans*

Class	N[d]	susptibility to [a]			uracil[b]	Eftomycin mg/ml[c]	
		FU	FUr	FUdR	(mM)	def	comp
MA2908		S	S	S	nd[e]	0.27	1.3
FUr^R	3	S	I	I	nd	0.26[f]	not done
FU^RI	4	R	R	S	nd	0.40	1.7
FU^RII	4	R	R	S	0.4	0.5	2.1
FU^RIII	4	R	R	S	1.1	0.5	2.2
FU^RIV	5	R	R	R	8.2	0.3	2.2
MA4820		R	R	R	5.0	<0.1	2.1

[a] S, MIC <= 10μg/ml; I, 10 μg/ml <MIC < 1 mg/ml; R, MIC > 1 mg/ml.

[b] peak extracellular uracil concentration in defined production medium (2–3 days).

[c] Seven days at 29°C in defined (def) or complex (comp) production medium.

[d] *N* = number of isolates.

[e] nd - none detected.

[f] only 1 of 3 isolates tested (FUr^R-1).

Table 2

Incorporation of exogenous uracil, uridine and adenine into TCA preciptable material by *N. lactamdurans* MA2908 and selected FU and FUr resistant mutants

Strain	gen time	Expt 1[a]			Expt 2
	(h)	nmol/mg protein[b]			nmol/mg protein
		adenine	uracil	U/A	uridine
MA2908	8.0	145.9	260.7	1.08	361.0
FUr^R-1	8.1	138.8	246.3	1.8	5.9
FU^R1-15 (I)[c]	8.3	68.4	24.3	0.4	24.3
FU^R1-44 (II)	8.3	39.5	9.3	0.2	67.9
FU^R1-40 (III)	8.2	45.8	2.7	0.06	49.0
FU^R1-24 (IV)	8.8	28.8	0.7	0.02	9.0
MA4820	8.1	46.7	0.9	0.02	26.1

[a] [2-^{14}C]-uracil and [2-^{3}H]-adenine present simultaneously.
[b] net incorporation from 30 to 150 min.
[c] class of mutant.

pathways. The incorporation of [2-^{14}C]-uracil and uridine into nucleic acids was determined. To control for non-specific effects on nucleic acid biosynthesis, the incorporation of uracil and [2-^{3}H]-adenine were determined simultaneously. The results in Table 2 represent net isotope incorporation into TCA precipitable material between 30 and 150 min, during which time incorporation is approximately linear (see Fig. 1). To a first approximation this incorporation represents RNA synthesis, since less than 10% of the radioactivity is associated with alkalai stable material (data not shown).

The FU^R mutants showed a reduced ability to incorporate both uracil and uridine that is coordinate with uracil excretion.This class of mutants also exhibit a marked reduction in adenine incorporation. Based on the ratio of uracil to adenine incorporation in dual label experiments, uracil incorporation is selectively inhibited. No ready explanation exists to explain the response to exogenous adenine, but is has been demonstrated that the incorporation of uracil and adenine are not mutually inhibitory (data not shown).

The fluorouridine resistant mutants that have been examined resemble FUr^R-1 in showing no reduction in uracil (or adenine) incorporation but a marked reduction in the ability to incorporate uridine.

The possibility of FU resistance resulting from loss of transport function was considered. Experiments of the type illustrated in Fig. 2 were used to evaluate uracil transport. The rate of uptake is linear for at least four minutes with no evidence of saturation. While the parameter we selected represents total uracil incorporation, during the four minute incubation less than 10% of the radioactivity is associated with TCA precipitable material. The maximum variability observed between independently prepared cell suspensions was 15%.

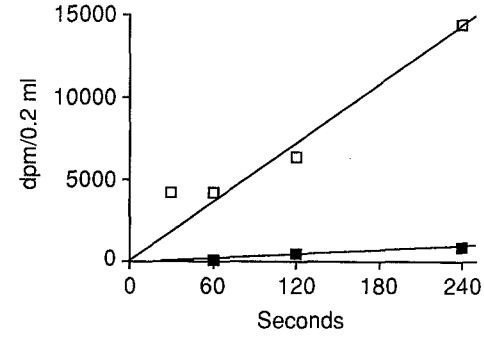

Fig. 2. Uracil transport by *N. lactamdurans* MA2908. Open squares, total incorporation; Solid squares, incorporation into TCA precipitable material.

With the exception of mutant FU^R 1-24, none of the mutants differed significantly from MA2908 with respect to uracil transport (Table 3). Even in this case, the observed difference is minor compared to the reduction of uracil incorporation into nucleic acids. Thus, it does not appear that the inability to transport uracil or uridine is sufficient to explain the poor incorporation of exogenous uracil and uridine into RNA observed in the FU resistant mutants.

Included in Table 3 are results from an independent experiment to determine the metabolic fate of the transported uracil. The fraction of radioactivity that could be accounted for by uridine nucleotides varied from 30 to 55%. No attempt was made to identify the remainder, however the migration rate on PEI cellulose was considerably faster than UMP. All of the FU resistant mutants are capable of converting exogenous uracil to the nucleotide level. A trend toward decreased incorporation into the UTP pool may exist in the FU resistant mutants. In the case of MA2908, approximately 10% of the radioactivity in the nucleotide pool comigrates with UTP. This value decreases to about 3% in MA4820 and FU^R 1-24.

Uridine is also transported by the FU resistant isolates, however, the FUr resistant isolate, FUr^R-1, is greatly inhibited in this regard. The rate of transport is only 1% of that observed for its parent, MA2908 (data not shown).

Table 3

Transport of [2-^{14}C]-uracil and its conversion into nucleotides by *N. lactamdurans* MA2908 and representative FU resistant mutants

Strain	uracil transport[a] pmol/min mg protein	^{14}C-U incorporation (pmol/mg protein) into uridine nucleotide pool[b]			
		Fc	UMP	UDP	UTP
MA2908	220	0.54	300	70	40
FUr^R-1	270	0.47	420	140	40
FU^R 1-15 (I)	180	0.36	220	30	10
FU^R 1-44 (II)	250	0.38	240	120	20
FU^R 1-40 (III)	170	0.43	140	40	10
FU^R 1-24 (IV)	100	0.32	100	70	<5
MA4820	250	0.54	240	80	10

[a] Average of two independent experiments.
[b] Incorporation after 4 min incubation in buffer, the results from a single experiment.
[c] Fraction of total radioactivity in the uridine nucleotide spots.

Elevated UMP pool in FU resistant mutants

If the size of the uridine nucleotide pool were elevated in the FU resistant mutants, it would be possible to reconcile the apparent contradiction between the incorporation of exogenous uracil into nucleic acids with the ability of the isolates to both transport and activate uracil at approximately parental rates. Expansion of the uridine necleotide pool due to an increase in the rate of biosynthesis would reduce uracil incorporation into macromolecules by simple dilution.

To investigate this possibility, cells harvested in mid-log phase were extracted with 1 M HCOOH as described in Materials and Methods and analyzed by ion-pair HPLC [19]. The peaks were quantitated by using external standards and were confirmed by cochromatography with known compounds. The results of two independent experiments are summarized in Table 4. As the progression through mutant classes I–IV was made the UMP pool was observed to increase approximately 10 fold, from 0.9 to 11.5 nmol/mg protein. A slight elevation was also noted in the UDP pool, but amounted to only a 1.6–2 fold increase for the class IV mutants, FU^R 1-24 and MA4820. No elevation of the UTP pool was detected and no discernible pattern existed with regard to the ATP pool.

Table 4

The uridine nucleotide pool of *N. lactamdurans* MA2908 and representative FU resistant mutants

Strain	pmol/mg protein[a]			
	UMP	UDP	UTP	ATP
MA2908	900	1500	1150	2400
FUR 1-15	1200	950	1250	1150
FUR 1-44	1850	1600	1350	1350
FUR 1-40	3250	2150	1050	1850
FUR 1-24	11550	2400	1150	2150
MA4820	9750	3000	1200	1400

[a] Average of two independent experiments.

DISCUSSION

This study was prompted by observations of high concentrations of uracil in the broths of suboptimal efrotomycin fermentations. The assumption was that overproduction of uracil was detrimental and that if the phenomenon could be eleminated efrotomycin productivity could be increased. As a first step it was deemed important to determine the basis of uracil accumulation. The seminal observation of these experiments, namely that the uracil overproducing mutants exhibit improved efrotomycin productivity, was unanticipated. No satisfactory explanation yet exists to explain this association.

The inability of MA4820 to incorporate either exogenous uracil or uridine into nucleic acids suggested a defect in the pyrimidine salvage pathway (reviewed by Neuhard [14]), an assumption that seemed even more reasonable when resistance to FU and FUr was observed in this strain, implying the inability to convert the 5-fluoro analogs to FUMP and ultimately FUTP [6]. In *Salmonella typhimurium*, this resistance pattern commonly requires two genetic lesions, loss of uracil phosphoribosyl transferase and uridine kinase [1].

By using 5-fluorouracil resistance as a selectable marker we were able to isolate four classes of mutants. The classification was based on the amount of uracil accumulated in fermentation broths; class I mutants representing non-excretors and class IV mutants accumulating levels of uracil in excess of 5 mM. MA4820 was indistinguishable from mutants placed in class IV. Uridine kinase activity could not be detected in MA2908, an observation not unique to this species [11,12,17]. The inability to detect this enzyme was consistent with the ease of mutant isolation since a single mutation resulting in the loss of uracil phosphoribosyltransferase would now result in resistance. The activity of this enzyme was measured in representatives of the four mutant classes and no differences from the parent could be detected with either uracil or fluorouracil as substrates (data not shown).

One interesting mechanism of FU and FUr resistance which was taken advantage of by O'Donovan and Gerhart [16], is based on the overproduction of uracil by derepression of the pyrimidine biosynthetic pathway. These authors were successful in developing resistance to both of these agents by screening for uracil excretion; conversely, uracil overproducers were isolated by direct isolation of FU + FUr resistant mutants. In two of the three classes of mutants pyrimidine biosynthetic enzymes were found to be derepressed. One of these was later defined as a UMP kinase mutant based on genetic and biochemical data [10]. The basis of FU and FUr resistance was essentially reduced to one of competition with the natural pyrimidine nucleotides. The other class of derepressed mutants has, to the best of our knowledge, not been defined.

The FU resistant mutants described above appear to be resistant by virtue of an expanded UMP pool. The 10 fold elevation of the UMP as well as more modest increases in the UDP pool suggest that resistance development may be a result of dilution of FUMP. Furthermore, an increase in nucleotide pool can explain the reduction in exogenous uracil and uridine incorporation into nucleic acids. Accumulation of uracil in the medium can then result from UMP catabolism. Data by Nielsen (personal communication) demonstrate derepression of the pyrimidine biosynthetic pathway.

Derepression of the pyrimidine biosynthetic pathway in *S. typhimurium* has resulted from mutations in three distinct loci. While none of these mechanisms can be rigorously excluded, neither do they appear totally satisfactory explanations of the above results. UMP kinase mutants (*pyrH*) have been studied in detail by Neuhard and coworkers [7,10]. A hallmark of these mutants is a reduction in the UTP pool. No such differences exist in the mutants described in this paper. The decrease in relative amount of label seen in the UTP pool (Table 3) could easily reflect the expansion of the UMP and UDP pools.

Purine metabolism has been implicated in the control of pyrimidine biosynthesis by virtue of isolation of a leaky *guaB* (IMP dehydrogenase) mutant of *S. typhimurium* [8]. Reduction of the GTP pool to 20% of normal accompanies this mutation. The limits of detection in the present paper were approximately 1 pmole of GTP/ 10 μl injection which cor-

responds to about 500 pmol/mg protein). Under these conditions no differences could be discerned between the mutants and parent (data not shown). Finally, Jensen and coworkers [9] have described a RNA polymerase mutant isolated by selection to FU and FUr which exhibit high constitutive levels of aspartate transcarbamylase and orotate phosphoribosyl transferase. As might be expected from mutants with altered RNA polymerase, these mutants had significantly increased generation times in both minimal and complex media. No differences in growth rate were observed with our mutants.

Among other questions that need to be addressed is the reason for the inhibition of adenine incorporation into nucleic acids. Nucleoside diphosphate kinase activity [5] has been examined and appears unaffected in these mutants. The phenomenon does not appear to be related to a general effect on growth and it has also been shown that uracil and adenine incorporation are not mutually inhibitory. Since the most commonly encountered resistance mechanism to FU in the enterics is the loss of uracil phosphoribosyl transferase, it is surprising that none of the spontaneous mutants we have investigated to date exhibit this phenotype. Finally, although a connection between efrotomycin productivity and uracil overproduction was anticipated, the nature of the relationship was unexpected. No simple explanation exists to explain the increased yield of efrotomycin seen in the fluoruracil-resistant mutants.

REFERENCES

1 Beck, C.F., J.L. Ingraham, J. Neuhard and E. Thomassen. 1972. Metabolism of pyrimidines and pyrimidine nucleosides by *Salmonella typhimurium* J. Bacteriol. 110:219–228.

2 Brockman, R.W., J.M. Davis and P. Stutts. 1960. Metabolism of uracil and 5-fluorouracil by drug resistant bacteria. Biochim. Biophys. Acta 40: 22–32.

3 Cromwell, G.L., H.J. Monegue and T.S. Stahly. 1985. Efficacy of efrotomycin as a growth promotant for swine. J. Anim. Sci. 61S: 313–314.

4 Foster, A.G. and D.L. Harris. 1976. Efrotomycin, a drug for swine dysentery control. J. Anim. Sci. 43: 252.

5 Ginther, C.L. and J.L. Ingraham. 1974. Nucleoside diphosphokinase of *Salmonella typhimurium*. J. Biol. Chem. 249: 3406–3411.

6 Horowitz, J. and E. Chargaff. 1959. Massive incorporation of 5-fluoro-uracil into a bacterial ribonucleic acid. Nature 184: 1213–1215.

7 Ingraham, J.L. and J. Neuhard. 1972. Cold-sensitive mutants of *Salmonella typhimurium* defective in uridine monophosphate kinase (*pyrH*). J. Biol. Chem. 247: 6259–6265.

8 Jensen, K.F. 1979. Apparent involvement of purines in the control of expression of *Salmonellas typhimurium pyr* genes: analysis of a leaky *guaB* mutant resistant to pyrimidine analogs. J. Bacteriol. 138: 731–738.

9 Jensen, K.F., J. Neuhard and L. Schack. 1982. RNA polymerase involvement in the regulation of expression of *Salmonella typhimurium pyr* genes. Isolation and characterization of a fluorouracil-resistant mutant with high, constitutive expression of the *pyrB* and *pyrE* genes due to a mutation in *rpoBC*. EMBO J. 1: 69–74.

10 Justesen, J. and J. Neuhard. 1975. *pyrR* identical to *pyrH* in *Salmonellas typhimurium* control of expression of the *pyr* genes. J. Bacteriol. 123: 851–854.

11 Jyssum, S. and K. Jyssum. 1979. Metabolism of pyrimidine bases and nucleosides in *Neisseria meningitides*. J. Bacteriol. 138: 320–323.

12 Kelln, R.A. and R.A.J. Warren. 1974. Pyrimidine metabolism in *Pseudomonas acidovorans*. Can. J. Microbiol. 20: 427–433.

13 Molley, A. and L.R. Finch. 1969. Uridine- 5'- monophosphate pyrophosphorylase activity from *Escherichia coli*. Fed. Eur. Biochem. Soc. Lett. 5: 211–213.

14 Neuhard, J. 1983. Utilization of preformed pyrimidine bases and nucleosides. In Metabolism of Nucleotides, Nucleosides and Nucleobases in Microorganisms. (Munch-Petersen, A., ed.) Academic Press. New York. 321 pp.

15 Nielsen, J. 1990. Coordinately elevated pyromidime biosynthetic enzymes in fluorouracil resistant mutants of *Nocardia lactamdurans*. J. Ind. Microbiol. 5:

16 O'Donovan, G.A. and J.C. Gerhart. 1972. Isolation and partial characterization of regulatory mutants of the pyrimidine pathway in *Salmonella typhimurium*. J. Bacteriol. 109: 1085–1096.

17 Ovrebo, S. and K. Kleppe. 1973. Pyrimidine metabolism in *Acinetobacter calcoaceticus* J. Bacteriol. 116: 331–336.

18 Parmeggiani, A. and G.W.M. Swart. 1985. Mechanism of action of kirromycin-like antibiotics. Ann. Rev. Microbiol. 39: 557–577.

19 Payne, S.M. and B.N. Ames. 1982. A procedure for rapid extraction and high-pressure liquid chromatographic separation of nucleotides and other small molecules from bacterial cells. Anal. Biochem. 123: 151–161.

20 Stapley, E.O., M. Jackson. S. Hernandez, S.B. Zimmerman, S.A. Currie, S. Mochales, J.M. Mata, H.B. Woodruff and D. Hendlin. 1972. Cephamycins, a new family of β-lactam antibiotics. I. Production by actinomycetes, including *Streptomyces lactamdurans* sp. n. Antimicrob. Agents Chemother. 2: 122–131.

21 Wax, R., W. Maise, R. Weston and J. Birnbaum. 1976. Efro-

tomycin, a new antibiotic from *Streptomyces lactamdurans*. J. Antibiotics 29: 670–673.

22 Wesseling, A.C. and B.D. Lago (1981). Strain improvement by genetic recombination of cephamycin producers, *Nocardia lactamdurans* and *Streptomyces griseus*. Dev. Ind. Microbiol. 22: 641–651.