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Coordinately elevated pyrimidine biosynthetic enzymes in fluorouracil resistant mutants of *Nocardia lactamdurans*

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SUMMARY

Nocardia lactamdurans, which produces the antibiotics efrotomycin and cephamycin C, exhibits essentially unregulated pyrimidine synthesis. Neither the first nor the second step is subject to feedback regulation. In a series of strains, comprising the original soil isolate, a set of spontaneous fluorouracil-resistant (FUR) mutants and the first improved efrotomycin producer, three of the pyrimidine biosynthetic enzymes were found to be coordinately regulated over a tenfold range. This amplification of enzyme activity accounts for the pyrimidine-related properties but not, at present, for their improved efrotomycin capacity.

INTRODUCTION

Strains of *Nocardia lactamdurans,* selected for spontaneous resistance to the pyrimidine analogue 5-fluorouracil (FU) [3], exhibit a range of properties related both to pyrimidine metabolism and the synthesis of the modified polyketide efrotomycin. Overall there is an approximately proportional relationship between the ability to excrete pyrimidines, chiefly as uracil, and the ability to synthesize efrotomycin. Pool sizes of pyrimidines and the activity of several salvage enzymes were compared in the series of strains, which include the original soil isolate, MA2908, selected for the production of the two antibiotics efrotomycin and cephamycin [7], the first improved strain in the efrotomycin genealogy and the FU-resistant series. Alterations in pyrimidine uptake, or in salvage pathways often responsible for the FUR phenotype could not account for resistance in the set of *N. lactamdurans* strains. In this paper the regulation and specific activities of enzymes in the de novo biosynthetic pathway to UMP are examined and are presented as an explantion of the FU-resistance phenotype.

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MATERIALS AND METHODS

$Na_2{}^{14}CO_3$ (16.4 mci/mmol) and ^{14}(COOH)-orotic acid (51.2 mci/mmol) were obtained from NEN. Other chemicals were obtained from Sigma.

Cell-free extracts for enzyme assay were prepared from approximately 24 h old cultures grown in chemically defined medium containing in g/l: glucose, 20; glycerol, 20; monosodium glutamate, 8.25; NH_4Cl, 2.0; aspartic acid, 0.5; NaCl, 0.5; K_2HPO_4, 1.33; $MgSO_4 \cdot 7H_2O$, 0.5; gelatin, 3; inositol, 0.2; $CaCO_3$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.025; $ZnSO_4 \cdot 7H_2O$, 0.010; $CuSO_4 \cdot 5H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 0.005; pH 7.0. Two ml of culture was harvested in microfuge tubes, the pellet washed three times with water and then sonicated in 0.5 ml of buffer for 30 s, 50% cycle at 50 W with chilling in salt-ice. The extraction buffer was 0.05 M HEPES pH 7.6, 20% glycerol, 0.1 M KCl, 0.05 mM EDTA, 2 mM dithiothreitol, and 1 mM diisopropylfluorophosphate (DFP). The DFP was added from 0.1 M stock solution in isopropyl alcohol just before sonication. Extracts were centrifuged for 5 min at 15 000 × *g*, and stored in ice until assay. Initially all the extracts were rapidly desalted by centrifugation through damp Sephadex G25 prior to assay, but it was found that this made no difference to specific activity for any of the enzymes assayed, and the step was eliminated.

Carbamyl phosphate synthase (CPS) was assayed using ^{14}C-Na_2CO_3 essentially as described in [5], trapping carbamyl phosphate with hydroxylamine. More than 95% of the label comigrated with hydroxyurea [2]. The standard reaction mix contained in 0.2 ml, 100 mM HEPES, 200 mM KCl, 36 mM $MgCl_2$, 24 mM ATP, 10 mM l-glutamine, 10 mM ^{14}C-Na_2CO_3 (200–400 dpm/mmol) and 1 mM reduced glutathione. The pH of the reaction mix was 7.6 at 30°C. Up to 20 µl of enzyme was added at zero time. After 15 min at 30°C 25 µl of 2 M hydroxylamine hydrochloride was added, the tubes incubated at 95°C for 10 min and the contents transferred to scintillation vials, acidified with 40 µl 5 N HCl and evacuated at 60°C to dryness in an oven fitted with a disposable trap containing Ascarite (A.H. Thomas). The vial contents were dissolved in 0.5 ml H_2O and 10 ml Scintiverse (Fisher) for counting at approximately 90% efficiency. As was described for the *Bacillus subtilis* enzyme, the activity obtained thus was 40–50% that obtained by trapping the carbamyl phosphate as carbamyl aspartate following the addition of aspartic acid in the presence of excess aspartate transcarbamylase (ATCase). However, as no convenient source of ATCase free of CPS, was available the less efficient but reliable hydroxamate trapping assay was used. The assay was linear with time at 30°C for 20–25 min and with enzyme up to about 20 nmol of product (10% of the carbonate). ATCase was assayed as described by Paulus and Switzer [6].

Carboxyl-labelled orotidine monophosphate (OMP) (190 dpm/nmol) was prepared from carboxyl-labelled orotic acid as described in [1]. After incubation of ^{14}C-orotic acid with yeast OMP pyrophosphorylase, the extent of reaction was monitored by ascending chromatography on PEI-cellulose developed in 0.3 M LiCl. 93% of the counts comigrated with OMP and less than 4% with orotate. OMP decarboxylase (OMPdc) was assayed by $^{14}CO_2$ trapping after incubation for 15 min at 30°C as described in [5] using this preparation without isolation of OMP. The use of carboxyl instead of ring labelled OMP as described in [1] is distinctly preferable because the $^{14}CO_2$ trapped measures all decarboxylation regardless of subsequent reaction to form uridine and uracil. 10 mM sodium arsenate inhibits dephosphorylation in crude extracts by about 90% but no fully inhibitory concentration could be found, necessitating the measurement of uracil, uridine and UMP, if ring labelled orotate is used. There is no single chromatographic system that resolves all three adequately from unreacted orotate. Freshly sonicated extracts were assayed in the presence of high concentrations of bovine serum albumin, as described by Paulus et al. [5].

RESULTS AND DISCUSSION

The specific activities of the first, second an last enzymes of de novo pyrimidine biosynthesis in the

soil isolate, MA2908, the series of spontaneous FUR strains and the improved efrotomycin producer MA4820 are shown in Table 1. Coordinate plots of aspartate transcarbamylase and OMP decarboxylase versus carbamyl phosphate synthase are shown in Figs. 1a and 1b respectively. There is an approximately coordinate relationship among the increases in enzyme activities in all the strains. MA4820, which was derived by several mutation steps from MA2908, falls close to the regression line established by the whole set.

Fig. 2 shows the relation of one enzyme (ATCase) to the internal uracil-derived pool, composed of UTP, UDP, UMP, and uridine (Fig. 2a) and to the excreted uracil level (Fig. 2b). It appears that any increase in ATCase is reflected in an increase in the internal pool, and that there is a nearly linear relationship between ATCase and the pool. When synthesis of pyrimidines is increased the pool expansion is predominantly in the form of UMP, with a much smaller increase in UDP and virtually none in UTP [3]. In contrast to pool size, the excretion of uracil (Fig. 2b) does not begin until more than a doubling of specific activity of ATCase has taken

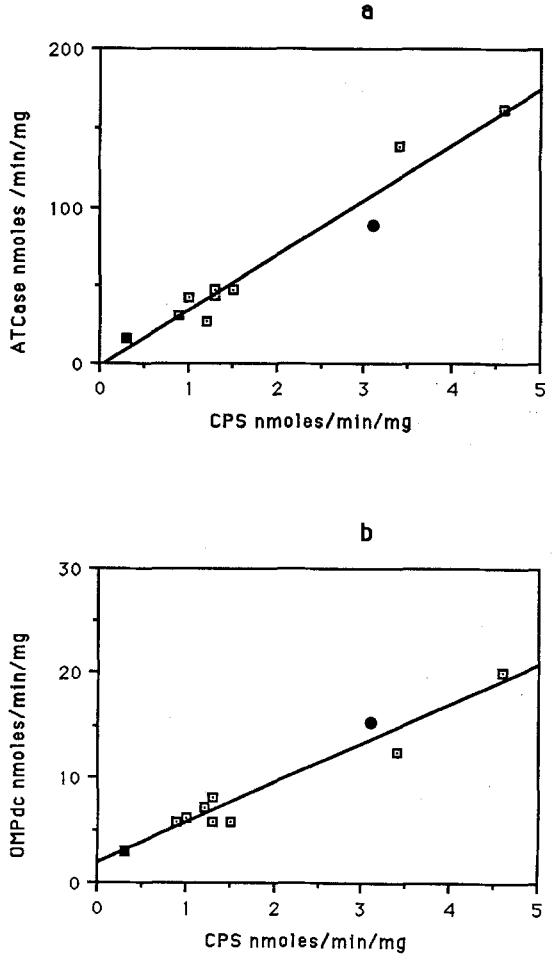

Fig. 1. Plot of the specific activities of (a) aspartate transcarbamylase versus carbamyl phosphate synthase, (b) OMP decarboxylase versus carbamyl phosphate synthase. The strains are MA2908, filled square: FUR strains, open squares: MA4820 filled circle. The units for all three enzymes are nmol/min/mg.

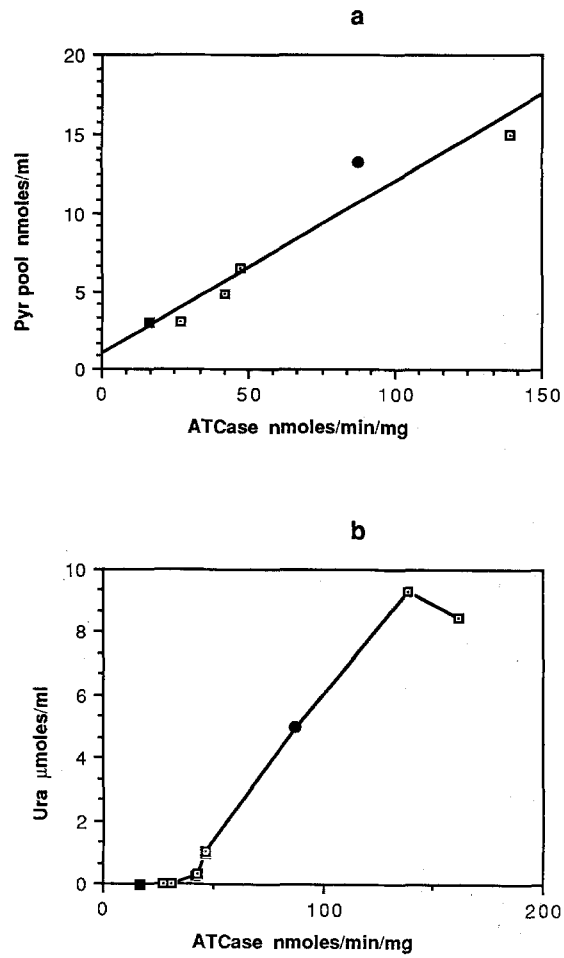

Fig. 2. Plot of the specific activity of aspartate transcarbamylase in nmol/min/mg versus (a) internal pyrimidine pool in nmol/ml culture [3], (b) excreted uracil in µmol/ml. The strains are MA2908, filled square: FUR strains, open squares: MA4820, filled circle.

Table 1

Pyrimidine enzyme specific activities and pool sizes

Strain	CPS (nm/min/mg)	ATCase (nm/min/mg)	OMPdc (nm/min/mg)	Uracil (μmol/ml)	Pyr. pool (nmol/ml)*
MA2908	0.3	16	3.0	0.00	3.0
FUR 1-15	1.2	27	7.1	0.00	3.0
FUR 2-25	0.9	31	5.7	0.00	nd
FUR 1-36	1.3	43	5.7	0.30	nd
FUR 1-44	1.0	42	6.2	0.26	4.8
FUR 1-22	1.5	47	5.8	0.97	nd
FUR 1-40	1.3	47	8.1	1.03	nd
FUR 1-24	3.4	139	12.4	9.30	6.5
FUR 2-33	4.6	162	20.1	8.50	15.1
MA4820	3.1	88	15.3	5.00	13.3

Specific activities of carbamyl phosphate synthase (CPS), aspartate transcarbamylase (ATCase) and OMP decarboxylase (OMPdc), extracellular uracil concentration and the intracellular pyrimidine pool (sum of UTP, UDP, UMP and uridine concentrations). nd = not done. * nmol/ml of culture, including extracellular volume.

place. CPS specific activity increases about fourfold before uracil excretion sets in (Table 1). All the FUR strains are elevated in the biosynthetic enzymes. One class of them, represented in the table by FUR 1-15, does not exhibit any detectible elevation in the pyrimidine pool, and does not excrete uracil. A second class, represented by FUR 1-44, with a two to three fold elevation in pyrimidine synthetic enzymes, exhibits a modest increase in internal pool size and a low level of uracil excretion. We conclude from the data presented here and from the absence of any measurable changes in FU transport into the cell or in uracil phosphoribosyl transferase activity, presented in the accompanying paper [3], that increased synthesis in an essentially unregulated system is responsible for the resistance to FU in the spontaneous mutants and in the improved efrotomycin producer MA4820, which also exhibits a FUR phenotype.

The carbamyl phosphate synthase of the original soil isolate (MA2908) appears to be neither repressed nor inhibited by pyrimidines. 5 mM UTP, UMP, uridine, uracil, CTP, CMP, cytidine or cytosine had no effect when added to the standard assay of desalted crude extract up to 5 mM. Arginine (10 mM) also had no effect, either by itself or in combination with a pyrimidine. Neither uracil nor arginine, or both, when added at the time of inoculation have any effect on the level of enzyme at 24 h, despite the presence of both throughout the growth period. Mutants of *N. lactamdurans* requiring both uracil and arginine can readily be found following UV or nitrosoguanidine mutagenesis. Two such auxotrophs revert spontaneously, always losing both requirements simultaneously. Thus, this organism appears to have a single carbamyl phosphate synthase providing carbamyl phosphate for both pyrimidine and arginine synthesis.

The aspartate transcarbamylase in MA2908 showed evidence of neither repression nor inhibition by pyrimidines. Thus the apparent complete lack of feedback control of pyrimidine synthesis at either the first or second step in the pathway in *N. lactamdurans* was present in the original soil isolate. Subsequent strains have remained unaltered in this regard. There is no obvious regulation of carbamyl phosphate synthase by arginine. The next step in arginine biosynthesis, however, that catalysed by ornithine transcarbamylase, does exhibit at least tenfold repression-derepression by arginine and ornithine (data not shown).

The six enzymes of de novo pyrimidine synthesis

in *B. subtilis* map in a cluster [3] and are coordinately regulated [4]. A series of spontaneous uracil-tolerant derivatives of a uracil sensitive strain, lacking the arginine repressible carbamyl phosphate synthase, exhibit a constant relative activity of all six biosynthetic enzymes over a tenfold range of derepression, strikingly similar to that reported here for the Gram-positive organism *N. lactamdurans*. However, *N. lactamdurans* differs from *B. subtilis* in having only one carbamyl phosphate synthase to provide both arginine and pyrimidines, and in exhibiting no evidence of feedback repression.

We have been unable to determine the connection between pyrimidine synthesis and improved productivity of efrotomycin, and are continuing to pursue a biosynthetic or genetic explanation. Elevation of pyrimidine synthesis at the levels exhibited by MA4820 has been retained throughout the efrotomycin genealogy subsequently derived from this strain.

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