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Biochemical and genetic studies of a histidine regulatory mutant of *Streptomyces coelicolor* A3 (2)

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The specific activities of three enzymes in a histidine regulatory mutant RF59 of *Streptomyces coelicolor* A3(2) resistant to the histidine analogue 1,2,4-triazolealanine (TRA) were measured and compared to the activity of the wild type strain. The first enzyme of the histidine pathway, phosphorybosyl-ATP-pyrophosphorylase (PR-ATP-pyrophosphorylase), of mutant RF59 and the wild type was sensitive to allosteric inhibition by L-histidine and hence feed-back inhibition was not affected by mutation, although the specific activity in the mutant was 2.9 fold higher than in the wild type.

The other two enzymes coded by genes from the histidine operon were significantly derepressed. The enzyme D-erythroimidazoleglycerol phosphate dehydrase (IGP-dehydrase) in mutant RF59 had a 4.9 fold higher specific activity than in the wild type strain. The specific activity of the last enzyme of the pathway, histidinol-dehydrogenase (Hol-dehydrogenase), in the mutant was 4.7 fold derepressed compared to the wild type strain.

The results of genetic crosses revealed the mapping of RF59 regulatory mutation between *arg*A1 and *cys*D18 on *S. coelicolor* chromosome, suggesting that the mutant RF59 is a regulatory mutant unable to fully repress genes of the histidine pathway.

Although the regulation of amino acid biosynthetic pathways in Eubacteria, particularly in *Escherichia coli* and *Salmonella typhimurium*, is experimentally well documented (GREENBERG 1969, UMBARGER 1969) this field of streptomycete biology has been neglected. The main reason is the fact that this group of microorganisms is notable for antibiotic production and hence secondary metabolism has received more attention than primary metabolism. The isolation and characterisation of amino acid regulatory mutants in streptomycetes is, therefore, lacking.

We isolated a histidine regulatory mutant in *Streptomyces coelicolor* A3(2) which accumulated L-histidine in the culture broth (DERKOS-SOJAK and DELIĆ 1981). This mutant was resistant to the histidine analogue 1,2,4-triazolealanine (TRA), suggesting the derepression of histidine biosynthetic genes (DERKOS-SOJAK *et al.* 1977).

Genetic mapping studies of histidine auxotrophs in *S. coelicolor* A3(2) have revealed that out of nine genes controlling ten steps of histidine biosynthesis, five (A, B, C, G, I) are clustered in a presumptive *his* operon, while four (E, H, D, F) are located elsewhere on the chromosome (HOPWOOD 1967, RUSSI *et al.* 1973). Here we describe derepression of the histidine operon in the regulatory mutant *S. coelicolor* RF59 based on studies of three enzymes of the histidine biosynthetic pathway, and mapping of a regulatory mutation. The first enzyme, PR-ATP-pyrophosphorylase, is coded by gene *his*H located clockwise of the his operon. The other two enzymes, IGP-dehydrase and Hol-dehydrogenase, are coded by genes *his*B and *his*A in the *his* operon.

The histidine regulatory mutation (RF59) has been mapped on the *S. coelicolor* A3(2) chromosome.

Materials and methods

Strains and techniques: Media and general techniques were those described by HOPWOOD (1967). *Streptomyces coelicolor* A3 (2) and its auxotrophic mutants (Table 1) were kindly supplied by Prof. D. A. HOPWOOD, John Innes Institute, Norwich, England.

The derepressed mutant 1404 RF59 (DERKOS-SOJAK *et al.* 1977), was isolated after mutagenesis of the *S. coelicolor his*B12 auxotroph with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) (DELIĆ *et al.* 1970).

By subsequent treatment of 1404 RF59 with NTG, the auxotrophic markers *ade*-40 and *trp*-30 were introduced, without affecting the first mutation. The new mutants and recombinants (Table 1) were used for genetic crosses by protoplast fusion as described by HRANUELI *et al.* (1983). The techniques used in the analysis of haploid recombinants were those described by HOPWOOD (1967).

Preparation of crude extracts: Minimal medium (MM) was inoculated with a heavy spore suspension and incubated for 48 h on a New Brunswick rotary shaker Model G25 at 230 rpm. Mycelium was collected by centrifugation at 3000 × g on a SORVALL Superspeed Centrifuge Model RC2-B, and washed twice with tris-hydroxy-methyl-amynomethane 0.1 M (tris-HCl) buffer, pH 7.5. Pellets were stored at −20 °C for long periods. Two g of wet mycelium were suspended in 5 ml of 0.1 M tris-HCl buffer pH 7.5, containing 0.01 M β-mercaptoethanol (MARTIN 1963), and 0.004 M ethylendiaminotetraacetic acid (EDTA).

The suspension was kept at room temperature for 2 h, and subjected to disruption by ultrasonic waves for 3 min in a "Biosonic" Model II (Bronwill Scientific Division of Will Scientific Inc., Rochester, New York) at an intensity of 80%. After disruption, the mixture was centrifuged at 27000 × g for 30 min. The resulting crude supernatants were partially purified on a 1 × 30 cm column of Sephadex G-50 (PHARMACIA, Uppsala, Sweden). One ml of extract was passed through the column with tris-HCl buffer, pH 7.5, and 1 ml fractions were collected and used for enzyme assays. Protein concentrations were measured by the method of LOWRY *et al.* (1951).

Enzyme assay: Phosphoribosyl-ATP-pyrophosphorylase activity was determined by the method of AMES *et al.* (1961) with some modifications. The incubation mixture contained: 0.5 ml enzyme extract; 0.1 M tris-HCl buffer, pH 7.5; 10 mM $MgCl_2$; 13.2 mM ATP; 0.66 mM phosphoribosyl-pyrophosphate (PRPP) in 0.1 mM EDTA (total volume 3 ml). The reaction was started by adding PRPP to the reaction mixture, and increase of optical density was followed spectrophotometrically on a HITACHI PERKIN ELMER spectrophotometer Model 124, at 290 nm against a blank containing all components except PRPP. One unit of enzyme activity was defined as a change in absorbance of 1.00/min.

Imidazolglycero-phosphate dehydrase (IGP-dehydrase) activity was determined by measuring the absorption of the imidazoleacetol phosphate (IAP) formed in alkaline solution. The method was modified from that of MARTIN *et al.* (1971). The assay mix contained: 0.1 M triethanolamine-HCl, pH 8.1; 15.2 M β-mercaptoethanol and 0.1 M $MgCl_2$.

A reaction mixture contained 0.9 ml of the assay mix and 100 µl of enzyme extract. The reaction was started by adding 0.1 M imidazoleglycerol phosphate (IGP) to the reaction mixture (total volume 3 ml), incubated for 30 min at 37 °C and stopped by the addition of 1.43 N NaOH.

The absorbancy was read at 290 nm against the blank with water in place of IGP. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 mM of IAP per hour.

Histidinol dehydrogenase activity was determined according to MARTIN *et al.* (1971) with slight modifications. The assay mixture contained: 0.4 ml enzyme extract; 0.5 M glycine, pH 9 adjusted with NaOH; 0.015 M histidinol to the reaction mixture (total volume 3 ml) and was incubated 10 min at 25 °C. An increase of absorbance was followed at 240 nm on the spectrophotometer against a blank without histidinol.

Table 1

Streptomyces coelicolor A3(2) mutants

Stock No.	Chromosomal markers	Culture collection
A317	*his*A1 *ura*A1 *str*A1	John Innes Institute, Norwich
M124	*pro*A1 *arg*A1 *cys*D18	John Innes Institute, Norwich
1398	*ade*40 *trp*A30 RF59	Research Institute, PLIVA, Zagreb

Results

Enzyme activity in Streptomyces coelicolor A3(2) and S. coelicolor RF59 and level of derepression

The maximum activities of PR-ATP-pyrophosphorylase and Hol-dehydrogenase in both strains were obtained in fraction 5 from the Sephadex G-50 column, while maximum protein concentration occurred in fraction 6 (Fig. 1a, b). IGP-dehydrase had maximum activity in fraction probably due to different molecular weight of this enzyme in relation to other enzymes. The enzyme specific activities in crude extracts of the mutant RF59 (Fig. 1b) were higher than in the wild type (A3(2) (Fig. 1a), the ratios being 2.9 for PR-ATP-pyrophosphorylase, 4.7 for Hol-dehydrogenase and 4.9 for IGP-dehydrase (Table 2).

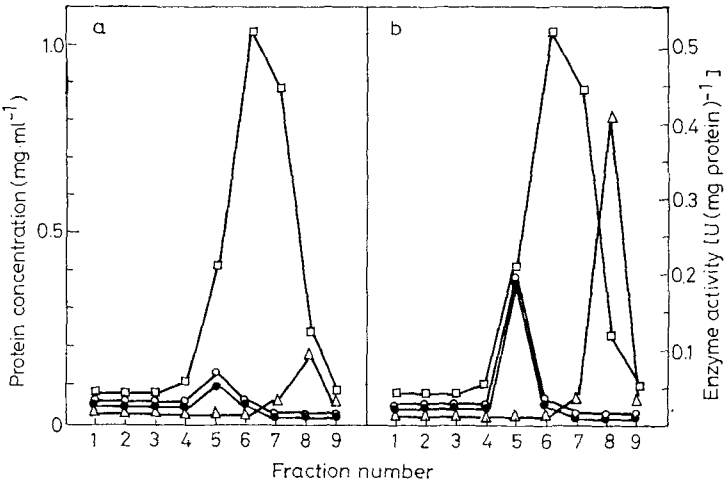


Fig. 1.

a) Concentration of protein and enzyme activities in *S. coelicolor* A3(2): □ concentration of protein (mg · ml^{-1}; ○ specific activity of PR-ATP-pyrophosphorylase [U(mg protein)$^{-1}$]; ● specific activity of Hol dehydrogenase [U(mg protein)$^{-1}$]; △ specific activity of IGP-dehydrase [U(mg protein)$^{-1}$].

b) Concentration of protein and enzyme activities in mutant *S, coelicolor* RF-59: □ concetration of proteinc (mg · ml^{-1}); ○ specific activity of PR-ATP-pyrophosphorylase [U(mg protein)$^{-1}$]; ● specific activity of Hol-dehydrogenase [U(mg protein)$^{-1}$]; △ specific activity of IGP-dehydrase [U(mg protein)$^{-1}$] .

Table 2

Ratio of specific activities of three enzymes in *S. coelicolor* A3(2) and *S. coelicolor* RF-59 [U(mg prot)$^{-1}$] Values are mean, ± standard error from five experiments.

Strain	PR-ATP-pyro-phosphorylase	Ratio	Hol-dehydro-genase	Ratio	IGP-dehydrase	Ratio
S. coelicolor A3(2)	0.065 ± 0.0040	2.9	0,040 ± 0.0012	4.7	0.085 ± 0.0002	4.9
S. coelicolor RF-59	0.194 ± 0.0044		0.190 ± 0.0029		0.422 ± 0.0023	

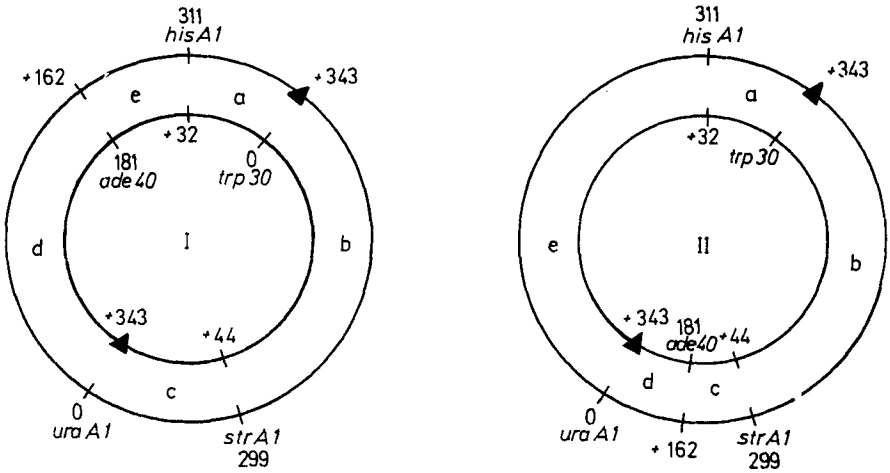
Inhibition of PR-ATP-pyrophosphorylase by L-histidine

Partially purified PR-ATP-pyrophosphorylase was tested for inhibition by various concentrations of histidine. Column fractions with maximum specific activities were used. As shown in Table 3, L-histidine at 10^{-2} M completely inhibited enzyme activity in both strains. L-histidine at 10^{-3} M gave 55—60% inhibition while inhibition at 10^{-4} M was only 25—35%. Inhibitions of mutant RF59 and the wild type A3(2) were not significantly different.

Table 3

Inhibition of PR-ATP-pyrophosphorylase activity in *Streptomyces coelicolor* A3(2) and *S. coelicolor* RF59 mutant by L-histidine. The results are average of five independent experiments.

Source of enzyme (strain)		Concentration of L-histidine			
		10^{-2} M	10^{-3} M	10^{-4} M	0.0 M
S. coelicolor A3(2)	Spec. activity	0.0	0.034	0.055	0.084
	% of inhibition	100.0	59.60	34.50	0.0
S. coelicolor RF59	Spec. activity	0.0	0.096	0.163	0.21
	% of inhibition	110.0	54.20	22.40	0.0



Genotype			Number	crossing-over in intervals	
				position I	position II
his	str	ade	129	c, d	c, e
+	+	+	0	a, b, d, e	a, b, c, d
his	+	ade	23	b, e	b, e
+	str	+	3	a, c, d, e	a, d
his	+	+	11	b, d	b, c, d, e
+	str	ade	19	a, c	a, c
his	str	+	148	c, d	d, e
+	+	ade	10	a, b	a, b

Fig. 2

Location of *ade*40 in a cross of A317 (*his*A1 *ura*A1 *str*A1) and 1398 (*ade*40 *trp*A30 RF59).

The patterns of crossing-over required to generate the observed recombinants on the two alternative locations are indicated in the table. Position I is chosen. Triangles in the diagrams indicate selected alleles.

Mapping of RF59 mutation on the Streptomyces coelicolor chromosome

By analysis of haploid recombinants the RF59 histidine regulatory mutation was shown not to map in or near the *his* operon. The positions of the newly introduced auxotrophic markers (Table 1) *trp*-30 and *ade*-40 were determined from the crosses of strain 1398 with A317 and M124 (Figs. 2 and 3). Thus *ade*-40 does not correspond to any known *ade* mutation, while *trp*-30 corresponds to *trp*A,B mutations. By using the same well-characterized mutants, the RF59 mutation was mapped between *arg*A1 and *cys*D18, but closer to *arg*A1, that is in a position outside the his operon and also far from *his*H (Fig. 3 and Table 4).

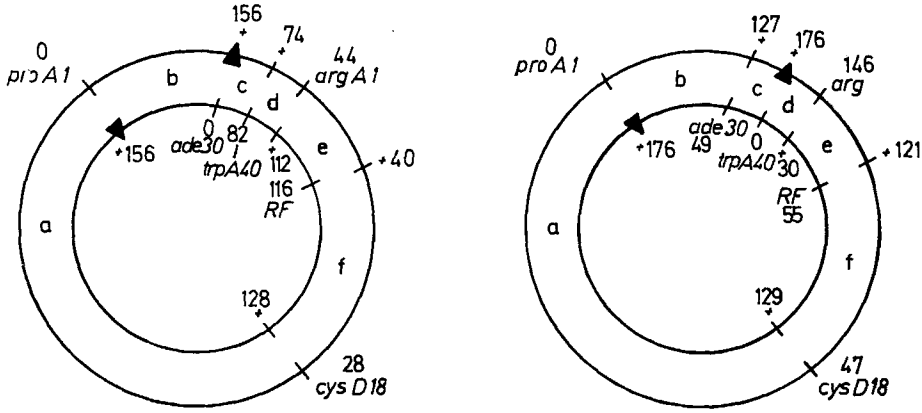


Fig. 3

Interpretation of the cross designed to determine the locations of *trp*A30 and RF59 mutations. Strains M124 (*arg*A1 *pro*A1 *cys*D18) (outer circles) and 1398 (*ade*40 *trp*A30 RF59) (inner circles) were crossed. Analysis of the segregation of RF59 in relation to that of *arg*A1 and *cys*D18 (see the 2 × 2 tabulations) indicate a position between *arg*A1 and *cys*D18.

The frequencies of individual recombinant genotypes on two selective media are given in Table 4.

	arg	arg$^+$	cys	cys$^+$		arg	arg$^+$	cys	cys$^+$
RF	17	99	4	112		29	37	3	33
RF$^+$	27	13	24	16		106	4	63	77
Selected alleles			pro A1$^+$/ade 30$^+$				pro A1$^+$/trp A 40$^+$		

	arg	arg$^+$	cys	cys$^+$		arg	arg$^+$	cys	cys$^+$
trp	3	79	6	76	ade	31	29	15	45
trp$^+$	41	33	22	52	ade$^+$	104	12	21	95
Selected alleles			pro A1$^+$/ade 30$^+$				pro A1$^+$/trp A40$^+$		

Discussion

Regulation of histidine biosynthesis in *Streptomyces coelicolor* A3(2) is partially under an operon control mechanism (Russi *et al.* 1973, Carere *et al.* 1973). The biochemical mechanism of regulation for this and other amino acids in streptomycetes has still

Table 4
Recombinant genotypes from the cross: M124 (*arg*A1 *pro*A1 *cys*D18) with 1398 (*ade* 40 *trp*A30 RF59)

recombinant genotypes				no.	crossovers required	recombinant genotypes				no.	crossovers required
cys	trp	arg	RF	0	a, b, c, d, e, f	cys	ade	arg	RF	0	a, c, e, f
+	+	+	+	0	b, d, e, f	+	+	+		0	b, d, e, f
cys	trp	arg	+	2	a, b, c, d	cys	ade	arg	+	14	a, c
+	+	+	RF	28	b, d	+	+	+	+	9	b, d
cys	+	+	+	5	a, b, d, e	cys	+	+	+	3	a, b, d, e
+	trp	arg	RF	0	b, d, d, e	+	ade	arg	RF	10	c, e
cys	trp	+	RF	2	a, b, c, f	cys	ade	+	RF	1	a, c, d, f
+	+	arg	+	9	b, f	+	+	arg	+	69	b, f
cys	+	arg	RF	2	a, b, e, f	cys	+	arg	RF	2	a, b, e, f
+	trp	+	+	6	b, c, e, f	+	ade	+	+	1	c, d, e, f
cys	+	+	RF	0	a, b, d, f	cys	ade	+	+	9	a, c, d, e
+	trp	arg	+	1	b, c, d, f	+	+	arg	RF	17	b, e
cys	trp	+	+	2	a, b, c, e	cys	+	arg	+	16	a, b,
+	+	arg	RF	15	b, e	+	ade	+	RF	27	c, d
cys	+	arg	+	15	a, b	cys	+	+	RF	0	a, b, d, f
+	trp	+	RF	69	b, c	+	ade	arg	+	7	c, f
Selected alleles				pro A1$^+$/ade 30$^+$		Selected alleles				pro A1$^+$/trp A40$^+$	

not been elucidated. A histidine regulatory mutant, RF59, which produces 3.5 g/l of L-histidine in the medium, compared with 0 g/l of the wild-type (DERKOS-SOJAK and DELIĆ 1981), is resistant to the L-histidine analogue TRA (DERKOS-SOJAK *et al.* 1977). *Salmonella typhimurium* regulatory mutants resistant to TRA showed derepressed enzyme levels for the histidine biosynthetic pathway (ROTH *et al.* 1966). It was thus possible that the RF59 regulatory mutant of *S. coelicolor* A3(2) was also derepressed at least for some enzymes of the histidine biosynthetic pathway.

We examined the specific activity of three enzymes of the pathway in partially purified crude extracts. The enzyme Hol-dehydrogenase, coded by the gene *his*A in the *his* cluster, was 4.7 fold derepressed in comparison to the wild-type A3(2), and IGP-dehydrase, coded by the gene *his*B also in the cluster, was 4.9 fold derepressed (Table 2). These values are not significantly different and indicate coordinate derepression of the two genes in RF59. PR-ATP-pyrophosphorylase, coded by gene *his*H located near the *his* cluster, but outside it, was also derepressed but to a lower extent (2.9 fold). RUSSI *et al.* (1973) showed that histidinol phosphate-phosphatase (HP-phosphatase), coded by the *his*D gene far from *his* cluster was not derepressed in the constitutive mutant *his*C 119-O$_c$, in contrast to two enzymes coded by genes in the *his* operon (*his*A and *his*B).

Since the RF59 mutation maps between *arg*A and *cys*D on the *S. coelicolor* chromosome (that is far from the structural genes studied), one repressor could exist affecting both the genes in and outside the operon but with different affinity.

Inhibition of partially purified PR-ATP-pyrophosphorylase (the first enzyme of the pathway) by various concentrations of L-histidine (Table 2) indicated that the enzyme of both strains, RF59 and A3(2), was equally sensitive to allosteric inhibition. Previous results on growth inhibition by the analogue 2-thiazole alanine (DERKOS-SOJAK *et al.* 1977) also suggested that the mutant is derepressed rather than being released from feed back inhibition. The results of derepression studies of the three enzymes, together with resistance of RF59 to the histidine analogue TRA (DERKOS-

SOJAK *et al.* 1977), indicate a repression control mechanism in the histidine biosynthetic pathway.

In *S. typhimurium* (ROTH *et al.* 1966) mutation in any of the five genes unlinked to the *his* operon can produce derepression. These are genes specifying histidyl-tRNA synthetase (*his*S) or histidyl-tRNA (*his*R, *his*T, *his*U, *his*W).

The characterisation of 1404 RF59 histidyl-tRNA or histidyl-tRNA synthetase should provide further information on histidine regulation in *S. coelicolor* A3(2).

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