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REVERSION OF THE EFFECTS OF A THREONINE DEAMINASE REGULATORY MUTANT BY A MUTATION IN ilvH IN ESCHERICHIA COLI K-12*

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In a strain carrying an ilvA538 mutation, the ilvGEDA operon expression is decreased (hyperattenuated) and the activity and/or expression of isoleucyl- and valyl- tRNA synthetases is decreased. We have isolated two revertants of ilvA538 owing to mutations in the ilvH gene, whose product is acetohydroxy acid synthase III. The regulatory properties of these revertants are consistent with a dual role for threonine deaminase as an effector of the ilvGEDA operon and the isoleucyl- and valyl- tRNA synthetase structural genes.

In both Escherichia coli (E. coli) and Salmonella typhimurium (S. typhimurium), the synthesis of valyl-, isoleucyl- and leucyl-tRNA synthetases (VRS, IRS, and LRS, respectively) is subject to a control process which may share signals with attenuation control for the isoleucine and valine biosynthetic genes. For the branched-chain amino acid-mediated control process, VRS formation is bivalently controlled by the supply of both valine and isoleucine, whereas IRS and LRS formation is in specific response to a growth rate-limiting supply of the respective branched-chain amino acid. (1,2) The results of several studies indicated that the ilvGEDA operon (Fig. 1) is regulated by an attenuation mechanism requiring each of the aminoacylated branched-chain amino acid tRNAS. (3-5)

Levinthal et al. (6) showed that a strain carrying the regulatory mutation, ilvA538, has lower than normal levels of all three branched-chain tRNA synthetases under conditions of excess isoleucine, valine and leucine and that synthesis in minimal medium was below the wild-type levels. The regulatory mutation also prevented a deattenuation response of the isoleucine and valine biosynthetic enzymes to limitations of branched-chain amino acids.

However, it was not clear whether threonine deaminase had a direct role in regulating synthesis of these enzymes. We have presented in vitro evidence for the inhibition of isoleucyl- and valyl-tRNA synthetase activities by threonine deaminase and 2-ketobutyrate, the product of the threonine deaminase reaction, through the formation of a high molecular weight complex of the three molecules (unpublished results). We proposed that threonine deaminase is a positive effector on ilvGEDA attenuation and that the complex formation is increased in an ilvA538 strain resulting in an increased inhibition of isoleucyl-and valyl-tRNA synthetase activities and thereby hyperattenuation of the ilv biosynthetic operons.

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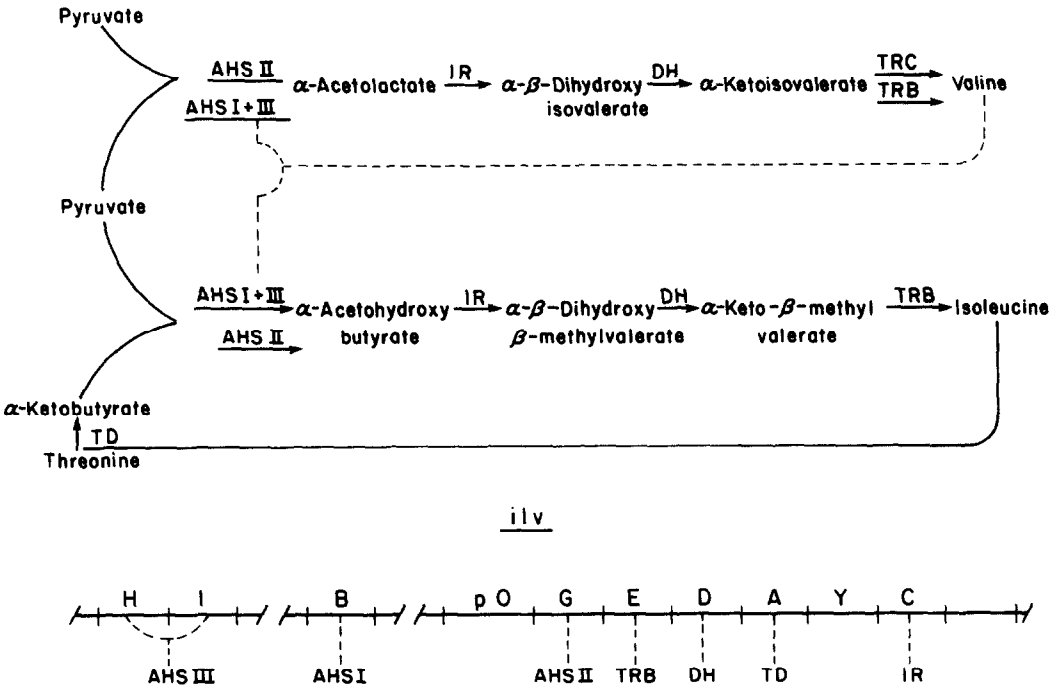

Figure 1. **Gene-enzyme relationships in branched-chain amino acid biosynthesis** (Top) Arrangement of the structural genes. AHS, Acetohydroxy acid synthase; IR, acetohydroxy acid isomeroreductase; DH, dihydroxy acid dehydrase; TRB, transaminase B.

In this report, we describe revertants of the ilvA538 mutant strain isolated as being growth resistant to the amino acid analog, valine hydroxamate. The revertants show an increase in expression and/or activities of the isoleucyl-and valyl-tRNA synthetases and essentially normal attenuation control of the ilvGEDA operon. This reversal of the ilvA538 phenotype is consistent with hypothesis that threonine deaminase functions as a positive effector in ilvGEDA attenuation and formation of a high molecular weight complex between threonine deaminase, 2-ketobutyrate, and isoleucyl-and valyl-tRNA synthetases.

EXPERIMENTAL

Bacterial Strains, Growth Media and Methods.

The E. coli K12 strains used are listed in Table 1. The phenotype of the ilvA538 mutation is an inhibition of growth by leucine (leu^S). The minimal medium used was the basal salts solution described by Fraenkel and Neidhardt (7) supplemented with 0.4% glucose and 0.2% ammonuim sulfate. Cells were grown at 37°C under constant aeration. Growth in excess branched-chain amino acids was achieved by incubation in minimal medium supplemented with L-valine(100μg/ml), L-isoleucine (50 μg/ml), and L-leucine (100μg/ml). We used the P1 CMts-100 phage and procedures of Rosner (8) for generalized transduction. Valine hydroxamate resistant strains were isolated without mutagenesis on gradient plates with a concentration range of 0 to 1.0 mg/ml valine hydroxamate. Two independent isolates were purified and shown to be resistant to 500 mg/ml of valine hydroxamate.

RESULTS

In E. coli K-12, strains carrying the regulatory mutation, ilvA538, are unable to increase the rate of synthesis of the branched-chain aminoacyl-tRNA synthetases and show

Table 1. Strain list

Strain	Genotype	Source or reference
PA101	ara rbs115 xyl-7 lacY1 malP ilvA538 ilvH865 HfrP053	Spontaneous valine hydroxamate (300 µg/ml) resistant derivative of PS1150
PA102	ara rbs115 xyl-7 lacY1 mglP ilvA538 ilvH866 HrfP053	Spontaneous valine hydroxamate (300 µg/ml) resistant derivative of PS1150
PA103	ara rbs115 xyl-7 lacY1 mglP ilvH865 HrfP053	P1 transduction of PA101 with PS1079 as donor
PA104	ara rbs115 xyl-7 lacY1 mglP ilvH866 HrfP053	P1 transduction of PA102 with PS1079 as donor
PA105	HfrC trpR thi-1 rbs115 bgl-32 fuc6 ilvB800::Mul ilvH865	P1 transduction of PS1456 with PA 102 as donor
PA106	HfrC trpR thi-1 rbs115 bgl-32 fuc-6 ivlB800::Mul ilvH866	P1 transduction of PS1456 with PA102 as donor
PA107	ilvD	EMS mutagenesis of PS1079
PA108	ilvD ilvA538	EMS mutagenesis of PS1150
PS1079	ara rbs115 xyl-7 lacY1 mglP HfrP053	M. Levinthal
PS1082	F⁻ ilvB800::Mul ilvH612 thi-1 ara trpR	M. Levinthal
PS1150	ara rbs115 xyl-7 lacY1 mglP ilvA538 HfrP053	M. Levinthal
PS1456	HfrC trpR thi-1 rbs115 Δ(ara leu ilvHI) bgl-32 ilvB800::Mul fuc-6	M. Levinthal
PS1897	ilvB800::Mul bgl-32 rbs115 thi-1 trpR fuc-6	M. Levinthal

hyperattenuated expression of the isoleucine-valine biosynthetic enzymes (6,9). The threonine deaminase from a strain carrying the ilvA538 mutation has lowered catalytic activity and is hypersensitive to inhibition by leucine and isoleucine (10). An ilvA538 containing strain is also more sensitive to valine hydroxamate, a competitive inhibitor of the valyl-tRNA synthetase, than is a wild-type ilvA+ strain (Fig. 2). Such strains can be used to isolate mutants that are resistant to valine hydroxamate and which show an increase in the levels of the valyl- and isoleucyl-tRNA synthetase. We isolated valine hydroxamate resistant (VHR) derivatives of PS1150 (ilvA538) which will grow in the presence of 500 µg/ml of valine hydroxamate in the growth medium. (Figure 2). Parenthetically, these valine hydroxamate-resistant strains retain their leucine-sensitive phenotype, which indicates that the ilvA538 allele is still present in the strain. We screened the valine hydroxamate-resistant strains for increases in the valyl-, isoleucyl-, or leucyl-tRNA synthetase activities as an indicator of altered synthetase control and selected strains for further study that had a 2- 2.5-fold increase in the valyl- and isoleucyl-tRNA synthetase activites (Table 2).

The levels of threonine deaminase and total AHAS in the ilvA538 VH^R strains were equal to the levels in the ilvA+ strains under culture conditions with excess leucine, isoleucine and valine in the medium (Table 3). In glucose-minimal medium grown cells, threonine deaminase and total AHAS activites in the ilvA538 VH^R strains were intermediate

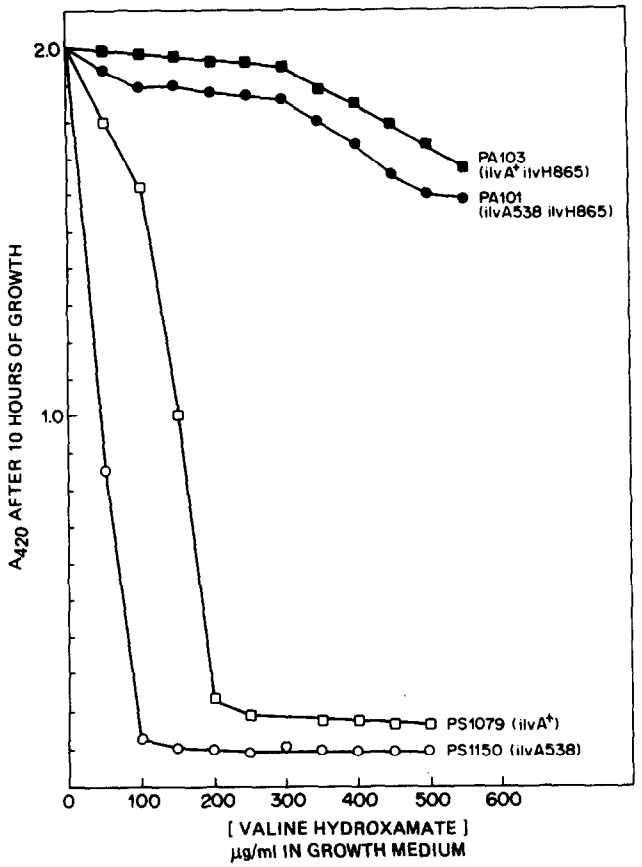

Figure 2. Growth of PS1079, PS1150, PA101, and PA103 in increasing concentrations of valine hydroxamate. Cells were grown overnight in glucose minimal medium, washed twice in minimal medium, and inoculated to an A_{420} of 0.1 in glucose minimal medium plus the indicated concentration of valine hydroxamate. The cultures were incubated at 37°C with aeration, and the A_{420} was measured after 10 hr of growth.

Table 2. Branched-chain aminoacyl-tRNA synthetase activities in valine hydroxamate-resistant strains

Strain	VRS	IRS	LRS
PS1079 (ilvA+)	0.21	0.13	0.17
PS1150 (ilvA538)	0.12	0.06	0.10
PA101 (ilvA538 ilvH865)	0.23	0.13	0.11
PA102 (ilvA538 ilvH866)	0.21	0.14	0.10
PA103 (ilvA+ ilvH865)	0.25	0.14	0.17
PA104 (ilvA+ ilvH866)	0.27	0.15	0.17

Crude extracts were made from cultures grown to mid-log phase in glucose minimal medium.
Specific activities are expressed as µmoles product/hr/mg protein-for valyl (VRS), isoleucyl (IRS) and leucyl (LRS)-tRNA synthetases.

Table 3. Isoleucine valine biosynthetic enzymes in valine hydroxamate-resistant strains

Strain	Growth conditions	Threonine deaminase	Acetohydroxy acid synthase
PS1079 *(ilvA+)*	MG	0.42	0.85
	LIV	0.32	0.35
PS1150 *(ilvA538)*	MG	0.22	0.46
	LIV	0.19	0.17
PA101 *(ilvA538 ilvH865)*	MG	0.34	0.58
	LIV	0.31	0.27
PA102 *(ilvA538 ilvH866)*	MG	0.34	0.66
	LIV	0.31	0.33
PA103 *(ilvA+ ilvH865)*	MG	0.34	0.86
	LIV	0.30	0.48
PA104 *(ilvA+ ilvH866)*	MG	0.36	0.77
	LIV	0.21	0.42

Sp. act. are expressed as umoles product/hr/mg protein.

MG = Glucose minimal medium
LIV = Glucose minimal medium plus leucine, isoleucine, and valine

between ilvA+ and ilvA538 strains. Furthermore, the VH^R mutation in an ilvA+ background does not alter the levels of the isoleucine-valine biosynthetic enzymes and the isoleucyl- and valyl-tRNA synthetases as compared to that of the wild type ilvA+ VH^S strains. Therefore, the ilvA538 VH^R strains show a phenotypic reversion of the hyperattenuated isoleucine-valine biosynthetic enzymes and the isoleucyl-and valyl -tRNA synthetases in an ilvA538 strain. After determining that the valine hydroxamate-resistant mutant was stable and exhibited elevated levels of isoleucyl-and valyl-tRNA synthetase (reversing the effect of ilvA538 on the isoleucyl-and valyl-tRNA synthetases but not leucyl-tRNA synthetase), we mapped the gene causing the valine hydroxamate-resistant phenotype. The VH^R phenotype was unlinked by P1 transduction to valS and 10% cotransducible to ileS.

In addition to being resistant to valine hydroxamate, these strains were also resistant to L-valine. (Fig. 3). Since valine resistance is a phenotype of an ilvH mutation which produces a valine resistant acetohydroxy acid synthase (AHAS) III, linkage of our mutations to the ilvH locus was examined. The mutation causing valine hydroxamate resistance was introduced into an ara leu ilvHI deletion strain, and in 250 transductants selected for Leu+ all exhibited valine hydroxamate resistance. For a total of 800 transductants tested in a variety of crosses, the valine hydroxamate resistant and valine resistant phenotypes were inseparable. Once deletion mapping indicated the close proximity between the gene causing valine hydroxamate resistant phenotype and the ilvH

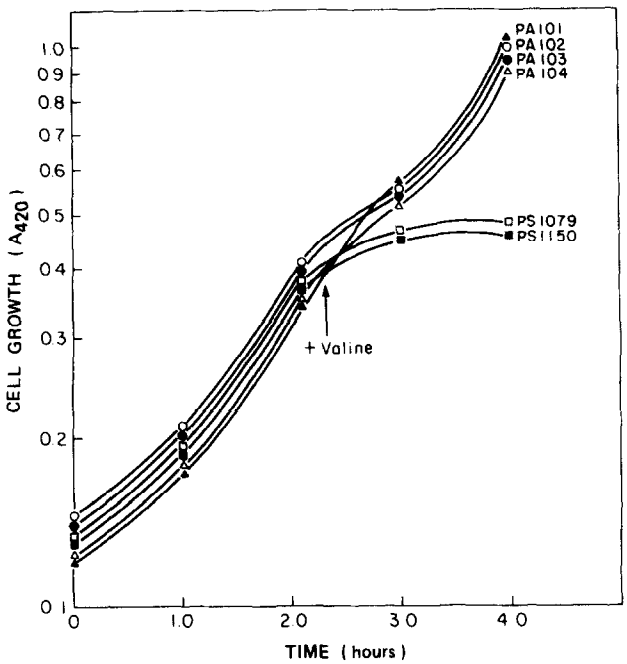

Figure 3. Growth of PS1079, PS1150, PA101, PA102, PA103, and PA104 in the presence of L-valine. The cells were grown in glucose-minimal medium overnight, washed twice in minimal medium, and inoculated into fresh glucose minimal medium to a A_{420} of 0.1 to 0.2. Growth was measured as A_{420} at the indicated times, and 100 μg/ml of L-valine was added as indicated.

locus, four point test crosses were devised to locate the gene more accurately (Table 4). The "valine hydroxamate resistance gene" was ordered with respect to carA, pdxA, and leuA. The gene causing a valine hydroxamate resistant phenotype was 50% co-transducible with carAB, 99% with pdxA, and 99% with leuA. The data does not allow discrimination between the orders pdxA leuA "VH^R" or pdxA "VH^R" leuA. However, all the data indicate that the locus causing the valine hydroxamate resistant phenotype is an allele of ilvH, implying the order pdxA leuA ilvH. Therefore, we designated two independently isolated mutations as ilvH865 and ilvH866.

To confirm this assignment, the sensitivity of the AHAS III activity to valine was examined in strains PA105 and PA106 (carrying ilvH865 and ilvH866). As controls, strains PS1082 (ilvH612, specifying a valine resistant AHAS III) and PS1897, an ilvH+ strain were examined (Fig. 4). All four strains also carried a mutation in ilvB so that the valine sensitive AHAS I activity was absent. The ilvH+ strain was sensitive to very low concentrations of valine in the reaction mixture, while the strain carrying the previously characterized ilvH612 allele was fully resistant to 0.6 mM valine in the reaction mixture. The valine hydroxamate resistant strains carrying the ilvH865 and ilvH866 alleles are just slightly less resistant to valine than the ilvH612 AHAS III mutant. Therefore, the mutation leading to valine hydroxamate resistance lies in the ilvH gene. In addition, crossfeeding and cross inhibition studies showed that the valine hydroxamate resistant strains were excreting valine into the medium and presumably contain high internal pools of valine.

DISCUSSION

In a strain carrying an ilvA538 mutation, the ilvGEDA operon expression is decreased (hyperattenuated) and the activity and/or expression of the isoleucyl- and valyl-tRNA synthetases is decreased. (6,9) We have isolated two revertants of the ilvA538 strain

Table 4. Four-point test cross to map the valine hydroxamate locus

Selected class	Number found with indicated phenotype	Phenotypes scored Car	Pdx	Leu	"VH^R"
Leu$^+$	4	-	-		-
	1	-	+		+
	25	+	+		-
	466	-	+		-
Pdx$^+$	1	+		-	+
	105	-		+	-
	29	+		+	-
Car$^+$	68		+	-	+
	81		+	+	-
	9		-	-	+

Probable order: carA pdxA (leuA "VH^R" is an allele of ilvH, then the probable order is carA pdxA leuA ilvH (VH^R).

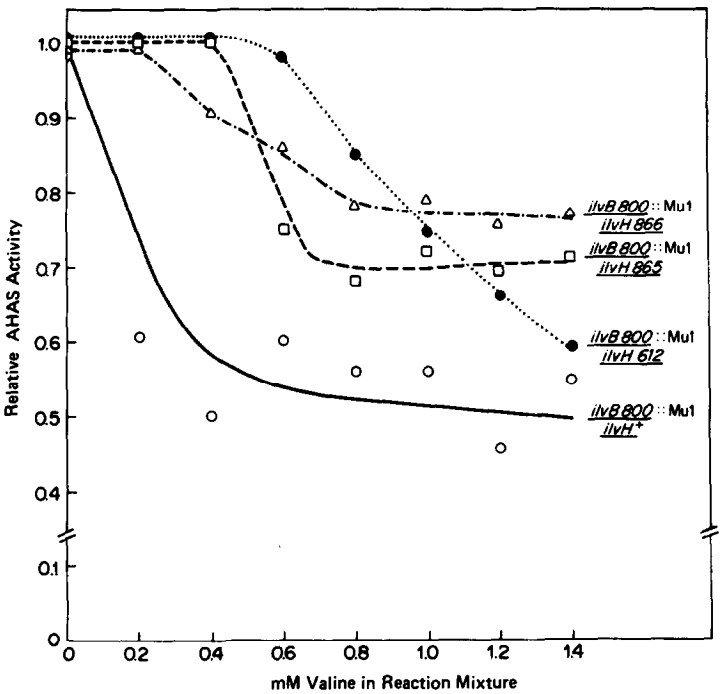

Figure 4. Measurement of valine-resistant AHAS III activities in PA101, PA103, PS1082, and PS1897. The cells were grown in glucose minimal medium overnight, washed twice in minimal medium, and inoculated in fresh glucose-minimal medium. Samples from each culture were taken at an A_{420} of 1.0, washed in potassium phosphate buffer plus isoleucine and pyridoxal phosphate, and resuspended in the same buffer. The enzyme extract was made via sonic disruption. The AHAS reaction was run for 20 min with the indicated concentration of valine in the reaction mixture. The AHAS activity of each sample was determined colorimetrically at A_{540}, and the relative AHAS activity was plotted versus the concentration of valine in the reaction mixture (mM). A relative activity of 1.0 is equivalent to a specific activity of 0.85 μM/hr/mg protein.

resistant to the amino acid analog, valine hydroxamate, which shows an essentially normal attenuation response of the ilvGEDA operon and increased activities of the isoleucyl-and valyl-tRNA synthetases. The reversion event is a mutation in the ilvH gene, which results in a valine resistant AHAS III.

We have recently observed a physical interaction between threonine deaminase, 2-ketobutyrate, and isoleucyl-and valyl-tRNA synthetases which is dependent upon the functional state of threonine deaminase. We proposed that the major regulatory role for threonine deaminase is direct action at the ilvGEDA leader region as an anti-attenuator. In addition, threonine deaminase can form an inactive complex with 2-ketobutyrate and isoleucyl- or valyl-tRNA synthetases. It is suggested that the formation of a high molecular weight complex blocks the anti-attenuator function of threonine deaminase, and inhibits the enzyme activity of the isoleucyl-and valyl-tRNA synthetases.

Since the site of the reversion mutation is in the ilvH gene (AHAS III) and is not directly involved involved in the formation of the high molecular weight complex, the ilvH gene product may be indirectly involved by altering the flow of carbon to isoleucine and valine. Nevertheless, the phenotype of this revertant supports the model for control of attenuation by threonine deaminase. The ilvH reversion restores apparently normal attenuation control to the ilvGEDA operon and increases the activities of the isoleucyl-and valyl-tRNA synthetases. The fact that the ilvGEDA operon and the tRNA synthetases are both affected by a single reversion event supports our model linking synthetase activities and ilvGEDA operon attenuation with the ilvA gene product, threonine deaminase.

In future studies we plan to isolate a series of ilvA538 revertants that are resistant to growth inhibition by leucine. If our model is correct, we should find additional classes of second site reversion mutations in ileS, valS, and the ilvGEDA leader region. We will also examine the effect of existing mutations in ileS and valS on threonine deaminase in ilvA+ or ilvA538 background.

REFERENCES

1. McGinnis, E. and L. S. Williams, (1971), J. Bacteriol. 108: 254-262.

2. McGinnis, E., A. C. Williams and L. S. Williams, (1974), J. Bacteriol. 252: 554-559

3. Blazey, D. L., R. Kim and R. O. Burns (1981) J. Bacteriol. 147: 452-462.

4. Lawther, R. P., and G. W. Hatfield, (1980), Proc. Natl. Acad. Sci. USA 77: 1862-1866.

5. Nargang, F. E., C. S. Subrahmanyam, and H. E. Umbarger, (1980). Proc. Natl. Acd. Sci. USA 77: 1823-1872.

6. Levinthal, M., M. Levinthal, and L. S. Williams, (1976). J. Mol. Biol. 102: 433-465.

7. Fraenkel, D. G. and F. C. Neidhardt, (1961) Biochim. Biophys. Acta 53: 96-110.

8. Rosner, J. L., (1972), Virology 49: 679-689.

9. Levinthal, M., L. S. Williams, M. Levinthal, and H. E. Umbarger. (1973) Nature (London) New Biol. 246: 65-68.

10. Calhoun, D. H. and G. W. Hatfield, (1975) Ann Rev. Microbiol. 29: 275-299.