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Regulatory Mutants of the *deo* Regulon in *Salmonella typhimurium*

James Blank and Patricia Hoffee

Department of Microbiology, School of Medicine, University of Pittsburgh
Pittsburgh, Pennsylvania

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Summary. A method is described for isolating mutants which are constitutive for thymidine phosphorylase. The mutants isolated are also constitutive for all of the enzymes of the *deo* regulon and are unlinked to the *deo* genes suggesting that they have a defect in a regulatory gene. We have designated this regulatory gene *deo R*.

In *Salmonella typhimurium* four enzymes involved in ribonucleoside and deoxyribonucleoside catabolism, i.e., deoxyribose-5-P aldolase (E.C. 4.1.2.4.), thymidine phosphorylase (E.C. 2.4.2.4.), phosphodeoxyribomutase (PDR mutase), and purine nucleoside phosphorylase (E.C. 2.4.2.1.) are coded for by the genes *deo C*, *deo A*, *deo B*, and *deo D*, respectively. These four closely linked genes are located on the chromosome counterclockwise to the *ser B* locus and have the genetic order *deo C—deo A—deo B—deo D* (Robertson *et al.*, 1970; Sanderson, 1970). The order of these genes appears to be identical to that found in *E. coli* (Ahmad and Pritchard, 1969).

In both *S. typhimurium* and *E. coli* the four genes appear to be regulated as two independent systems (Ahmad and Pritchard, 1969, 1971; Hammer-Jespersen *et al.*, 1971; Munch-Petersen, 1968a, 1968b; Robertson *et al.*, 1970). Deoxyribose-5-P aldolase and thymidine phosphorylase are induced by deoxyribose-5-phosphate (Barth *et al.*, 1968; Breitman and Bradford, 1967, 1968; Hoffee, 1968a; Hoffee and Robertson, 1969; Karlstrom, 1968) and are specific for deoxyribo-derivatives (Friedkin and Roberts, 1954; Hoffee, 1968b; Razzel and Casshyap, 1964). PDR mutase and purine nucleoside phosphorylase are induced by deoxyribose-5-phosphate and the purine ribonucleosides, guanosine and inosine and have specificity for both ribo- and deoxyribo-derivatives (Hammer-Jespersen *et al.*, 1971; Kammen and Koo, 1969; Karlstrom, 1968; Robertson *et al.*, 1970).

Previously we described some mutants defective in purine nucleoside phosphorylase (*deo D*) which show a polar effect on *deo B* but not on *deo A* or *deo C*. From this finding and the data from the induction patterns, we suggested that the *deo B* and *deo D* genes constitute a single operon and that the *deo C* and *deo A* genes may constitute a second closely linked operon. A similar hypothesis has been proposed for *E. coli* (Ahmad and Pritchard, 1969, 1971; Hammer-Jespersen, 1971; Munch-Petersen, 1968b).

In this paper we describe a method for isolating regulatory mutants in the *deo* system based on the competitive inhibition of thymidine phosphorylase by uridine. Many of these regulatory mutants have constitutive levels of all four of

the enzymes coded for by the *deo* genes suggesting that both operons are under the control of a single regulatory gene. We have designated this regulatory gene *deo R*.

Materials and Methods

Bacterial Strains. The bacterial strains employed in this study and their genotypes are listed in Table 1. Cultures were maintained on nutrient agar slants containing either 20 μg of thymine per ml or 50 μg of thymidine per ml. Constitutive strains were maintained on minimal glucose slants containing 2 μg thymine/ml and 1000 μg uridine/ml.

Materials. Commercial preparations were used unless otherwise specified. 2-Deoxyribose was obtained from International Chemical and Nuclear Corp., City of Industry, California. 2-Deoxyribose-5-phosphate, 2-deoxyribose-1-phosphate, glucose-1,6-diphosphate, inosine, thymine, thymidine, and uridine were purchased from Calbiochem, Los Angeles, California. Yeast alcohol dehydrogenase and xanthine oxidase were obtained from Boehringer and Sons, New York, New York. Crystalline deoxyribose-5-P aldolase was prepared from *S. typhimurium* (Hoffee, 1968b).

Culture and Growth of Organisms. Cells were grown as described previously (Hoffee and Robertson, 1969). Carbon sources were added to a final concentration of 0.2% when solid medium was used. Glucose low thymine-uridine medium contained 2 μg of thymine and 500 or 1000 μg of uridine per ml.

Induction of Enzymes. When wild-type cells were induced by purine nucleosides, the inducer was added to the cells (final concentration, 0.05%) during logarithmic growth in Casamino Acids. After 90 min in the presence of the inducer, the cells were harvested. When deoxyribose was used as the inducer (final concentration 0.2%), cells were harvested after 90% of the deoxyribose was utilized (about 2 hours). Constitutive mutants were grown overnight in Casamino Acids medium containing thymine (10 μg/ml) and harvested the next day. All cells were washed in 10^{-3} M ethylenediaminetetraacetate (EDTA), pH 7.4, and stored at −10° C.

Preparation of Extracts. Extracts of cells were made by grinding frozen pellets with alumina (twice the wet weight of the cells). The mixture was suspended in 10^{-3} M EDTA plus 10^{-2} M mercaptoethanol, pH 7.4, and centrifuged for 45 minutes at 27000 × *g*. The supernatant fluids were assayed for enzyme activities and protein concentration.

Enzyme Assays. Deoxyribose-5-P aldolase (Hoffee, 1968a), thymidine phosphorylase (Hoffee, 1968a), PDR mutase (Hoffee and Robertson, 1969), and purine nucleoside phosphorylase (Robertson *et al.*, 1970) were assayed as previously described.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951).

Isolation of Thymidine Phosphorylase Constitutive Mutants. Mutants constitutive for thymidine phosphorylase were derived from strains defective in PDR mutase by plating on minimal glucose plates containing thymine (2 μg/ml) and uridine (500 μg/ml). After several days of incubation at 37° C, resistant colonies (see Results) were picked and purified on minimal glucose media containing thymine (2 μg/ml) and uridine (1000 μg/ml). The auxotrophic strains required the addition of serine (20 μg/ml) or histidine (20 μg/ml) to the media.

Transduction Studies. Transduction studies were performed with overnight cultures of the various recipients and phage PLT 22 as previously described (Hoffee and Robertson, 1969).

Results

Isolation of Constitutive Mutants. Growth of a low thymine requiring mutant (*deo* B^-) is completely inhibited on glucose-thymine plates containing 500–1000 μg of uridine/ml. This inhibition is apparently due to a competitive inhibition of thymidine phosphorylase by uridine (Budman and Pardee, 1967; Yagil and Rosner, 1970). Mutants can overcome the inhibition by increasing the levels of thymidine phosphorylase in the cell, and then utilizing the external thymine for growth.

Deo B^- mutants (PH 4017, JB 3084, JB 3086, and JB 3094) were plated on glucose-thymine plates containing 500 μg uridine/ml. Resistant colonies were

Table 1. Bacterial strains

Strain	Genotype[a]					Other Characteristics[b]	Parental strain and origin
	deo A	*deo B*	*deo C*	*deo D*	*deo R*		
WT LT-2	+	+	+	+	+		E. Englesberg
PH 4008	+	+	−	+	+	*thy A*⁻	Hoffee and Robertson (1969)
PH 4017	+	−	+	+	+	*thy A*⁻	P. Hoffee
PH 4138	+	+	−	+	+	*thy A*⁻	P. Hoffee
JB 3000[c]	−	+	−	+	+	*thy A*⁻	Present study derived from PH 4008 (Robertson *et al.*, 1970)
JB 3004[c]	−	+	−	+	+	*thy A*⁻	Present study derived from PH 4008 (Robertson *et al.*, 1970)
JB 3051	+	−	+	+	c	*thy A*⁻	Present study derived from PH 4017
JB 3052	+	−	+	+	c	*thy A*⁻	Present study derived from PH 4017
JB 3054	+	−	+	+	c	*thy A*⁻	Present study derived from PH 4017
JB 3058	+	−	+	+	c	*thy A*⁻	Present study derived from PH 4017
JB 3084	+	−	+	+	+	*thy A*⁻ *his*⁻	Hfr A (Sanderson, 1970)
JB 3086	+	−	+	+	+	*thy A*⁻ *his*⁻	Hfr A (Sanderson, 1970)
JB 3089	+	−	+	+	+	*thy A*⁻ *his*⁻	Hfr A (Sanderson, 1970)
JB 3094	+	−	+	+	+	*thy A*⁻ *his*⁻	Hfr A (Sanderson, 1970)
JB 3107[d]	+	−	+	+	c	*his*⁻	Present study derived from JB 3084
JB 3108	+	−	+	+	c	*thy A*⁻ *his*⁻	Present study derived from JB 3086
JB 3162	+	+	+	+	c	*thy A*⁻ *his*⁻	Present study derived from JB 3108
JB 3164	+	+	+	+	c	*his*⁻	Present study derived from JB 3107
JB 3184	+	−	+	+	c	*thy A*⁻ *his*⁻	Present study derived from JB 3086
JB 3189	+	−	+	+	ts	*thy A*⁻ *his*⁻	Present study derived from JB 3094
JB 3190	+	−	+	+	ts	*thy A*⁻ *his*⁻	Present study derived from JB 3094
JB 3232	+	−	−	+	+	*thy A*⁻	Present study derived from PH 4138

[a] The following symbols are used: +, wild-type; −, genetic defect; *thy A*, thymidylate synthetase; *ser*, serine; *his*, histidine; *deo*, deoxyribonucleoside; *deo A*, thymidine phosphorylase; *deo B*, PDR mutase; *deo C*, deoxyribose-5-P aldolase; *deo D*, purine nucleoside phosphorylase; *deo R*, regulatory gene which can be expressed as inducible (+), constitutive (c) or temperature sensitive (ts).

[b] Thymine-requiring strains were isolated by a modification of the aminopterin method of Okada *et al.* (1966) as described previously (Hoffee, 1968a).

[c] This mutant was selected by plating PH 4008 on minimal glucose media containing 0.05% thymidine. Growth of *deo C* mutants are inhibited by thymidine (Robertson *et al.*, 1970). Colonies arising on these plates were tested on minimal-glucose plates containing either 20 μg of thymine or thymidine per ml. Those growing on the latter but not the former were isolated and purified.

[d] JB 3107 has reverted in *thy A*.

picked and purified on glucose-low thymine+uridine (1000 μg/ml) plates. The uninduced enzyme levels found in some of these mutants are shown in Table 2. All of the mutants listed are still *deo B*$^-$ but now have high constitutive levels of thymidine phosphorylase, DR-5-P aldolase and purine nucleoside phosphorylase.

In order to see if these mutants were constitutive for all four enzymes, two constitutive mutants (JB 3107, JB 3108) were transduced to *deo B*$^+$ using wild-type phage (Table 3). Two transductants, JB 3162 derived from JB 3108 and 3164 derived from JB 3107 are now *deo B*$^+$ and also constitutive for all four enzymes.

Transduction of the Constitutive Marker. P22 phage were grown on constitutive mutants and used to transduce the *deo A* and *deo C* markers. Table 4 shows the results of these transductions. When *deo C*$^-$ *deo A*$^-$ recipients were used, 0/97 and

Table 2. Enzyme levels in wild-type and constitutive strains of *Salmonella typhimurium*

Strain	Addition to medium[a]	DR-5-P aldolase[b]	Thymidine phosphorylase[b]	PDR mutase[b]	Purine nucleoside phosphorylase[b]
		Units/mg protein			
WT LT-2	None	40	70	50	80
	Deoxyribose	710	960	510	730
	Inosine	32	35	350	660
JB 3051	None	1850	5180	< 0.02	2020
JB 3052	None	1900	5300	< 0.02	1600
JB 3054	None	2540	3370	< 0.02	1070
JB 3058	None	3000	5000	< 0.02	2700
JB 3184	None	1160	2080	< 0.02	1230

[a] The strains were grown and induced as described in Materials and Methods.

[b] Alumina-ground extracts were assayed spectrophotometrically for deoxyribose-5-P aldolase (DR-5-P aldolase) and phosphodeoxyribomutase (PDR mutase). The reaction mixture for deoxyribose-5-P aldolase contained in 1 ml: 36 μmoles of triethanolamine buffer, pH 7.6, containing 1.0 μmole M EDTA; 0.1 μmole of NADH; 0.03 mg of alcohol dehydrogenase; and 0.5 μmole of deoxyribose-5-phosphate. A control without deoxyribose-5-phosphate was run with each assay. One unit of deoxyribose-5-P aldolase is defined as the number of nanomoles of deoxyribose-5-phosphate split per minute at 25° C. The 1 ml reaction mixture for PDR mutase contained: 40 μmoles of triethanolamine buffer, pH 7.8, 0.9 μmole of EDTA, 0.1 μmole of NADH, 1.0 μmole of $MnCl_2$, 0.5 nmoles of glucose-1,6-diphosphate, 420 units of purified deoxyribose-5-P aldolase, 0.03 mg of alcohol dehydrogenase, and 0.9 μmole of deoxyribose-1-phosphate. A control without deoxyribose-1-phosphate was run with each assay. One unit of PDR mutase is defined as the number of nanomoles of deoxyribose-1-phosphate converted to deoxyribose-5-phosphate per minute at 25° C.

The reaction mixture for thymidine phosphorylase contained in 0.2 ml: 3.7 μmoles of thymidine, 40 μmoles of tris (hydroxy-methyl) aminomethane chloride buffer, pH 7.4, and 20 μmoles of Na arsenate. The reaction was run at 37 °C, and samples were removed at 5 minute and 10 minute intervals; 1 ml of 0.3 N NaOH was added to stop the reaction. The increase in optical density was measured at 300 mμ (Friedkin and Roberts, 1954). One unit is defined as the number of nanomoles of thymine formed per minute at 37° C.

Purine nucleoside phosphorylase was assayed by the coupled xanthine oxidase method of Kalcker (1947). The reaction mixture of 1 ml contained: 0.5 μmoles of inosine; 50 μmoles of potassium phosphate buffer, pH 7.5; and 0.02 enzyme units of xanthine oxidase. The increase in absorbance which is due to the formation of uric acid was measured at 293 mμ. One unit is defined as the number of nanomoles of inosine split per minute at 25° C.

Table 3. Enzyme levels in *deoB*$^-$ and *deoB*$^+$ constitutive strains of *Salmonella typhimurium*[a]

Strain	DR-5-P aldolase	Thymidine phosphorylase	PDR mutase	Purine nucleoside phosphorylase
	Units/mg protein			
JB 3108	1150	2320	< 0.02	509
JB 3162	1000	3500	700	1600
JB 3107	1160	2320	< 0.02	518
JB 3164	1500	2900	1100	1900

[a] These strains were grown overnight in Casamino Acids medium containing thymine (10 μg/ml). The enzymes were assayed as described in Table 2.

Table 4. Test of genetic linkage between the *deo* genes and constitutivity

Cross	Donor phage genotype	Recipient genotype	Selected markers[a]	Unselected markers	No. of colonies tested for constitutivity[b]	Linkage[c] (%)
(1)	*deo B*$^-$ *deo R*^{c} (JB 3058)	*deo C*$^-$ *deo A*$^-$ (JB 3000)	*deo A*$^+$ *deo B*$^-$	*deo R*	97	0
(2)	*deo B*$^-$ *deo R*^{c} (JB 3108)	*deo C*$^-$ *deo A*$^-$ (JB 3004)	*deo A*$^+$ *deo B*$^-$	*deo R*	119	0
(3)	*deo B*$^-$ *deo R*^{c} (JB 3052)	*deo C*$^-$ *deo B*$^-$ (JB 3232)	*deo C*$^+$ *deo B*$^-$	*deo R*	55	0

[a] DR-5-P aldolase positive transductants (*deo C*$^+$) were selected on enriched deoxyribose plates containing 20 μg/ml thymine. Thymidine phosphorylase positive transductants (*deo A*$^+$) were selected on minimal glucose plates containing 20 μg/ml thymine. The *deoA*$^+$ transductants and the *deoC*$^+$ transductants were then tested for the *deoB*$^-$ marker. *deoB*$^-$ transductants give no growth on minimal thymidine plates.

[b] The *deo R* marker was tested by streaking transductants on minimal glucose plates containing 2 μg/ml thymine and 1000 μg/ml uridine and on minimal glucose plates. Those transductants growing on the thymine-uridine containing plate but not on the minimal glucose plates were scored as *deo R*^{c}. Only transductants which are *deoB*$^-$ can be tested by this method.

[c] Per cent linkage is defined as the number of *deo R*^{c} transductants/*deoC*$^+$*deoB*$^-$ colonies tested or/*deoA*$^+$*deoB*$^-$ colonies tested × 100.

0/119 of the *deoA*$^+$ *deoB*$^-$ transductants in crosses 1 and 2 respectively were constitutive for thymidine phosphorylase. When a *deoC*$^-$ *deoB*$^-$ recipient was used, 0/55 of the *deoC*$^+$ transductants were constitutive for thymidine phosphorylase. The data from these crosses indicate that the constitutive marker for the three mutants tested is not linked to the *deo* genes, and is probably not an operator type of mutation. Using the selection procedure described in Materials and Methods, over 40 mutants have been isolated. All of the mutants tested (about 50%) have constitutive levels of thymidine phosphorylase, DR-5-P aldolase, and purine nucleo ide phosphorylase.

Isolation of Temperature Sensitive Mutants Constitutive for Thymidine Phosphorylase. Temperature sensitive constitutive mutants were isolated by plating a

Table 5. Levels of *deo* enzymes in temperature sensitive mutants

Strain	Temperature of growth	DR-5-P aldolase	Thymidine phosphorylase	PDR mutase	Purine nucleoside phosphorylase
		Units/mg protein[a]			
JB 3189	25°	100	300	20	200
	42°	1900	2300	50	1000
JB 3190	25°	200	300	20	200
	42°	1300	3600	< 0.2	1000

[a] Strains were grown overnight in Casamino Acids medium containing thymine (10 μg/ml) at the indicated temperature. Enzyme assays were performed as described in Legend to Table 2.

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deo B$^-$ strain JB 3094 on glucose-low thymine plates containing uridine plus histidine and incubating at 42° C. Colonies growing at 42° C were tested for growth at 25° C on glucose-thymine-uridine-his plates. Two strains JB 3189 and JB 3190 were able to grow on these plates at 42° C but not at 25° C. Growth on glucose-thymine-his plates was normal at both temperatures. JB 3189 and JB 3190 had high constitutive levels of three of the *deo* enzymes at 42° C but relatively low levels at 25° C (Table 5). Even at 25° C the levels are higher than in unindiced wild-type strains (Table 3). Both of these mutants appear to have leaky levels of PDR mutase about 5–15% of expected levels. They still, however, are low thymine-requiring mutants. The uninduced levels of the *deo* enzymes in wild-type cells grown at 25° C and 42° C do not differ from that shown in Table 3 (data not shown).

Discussion

In this paper we describe a method for isolating mutants which are constitutive for thymidine phosphorylase. The selection procedure is based on the competitive inhibition of thymidine phosphorylase by uridine in low thymine-requiring strains. Low thymine-requiring mutants of *E. coli* and *S. typhimurium*, in addition to missing thymidylate synthetase, are deficient in either deoxyribose 5-P aldolase or PDR mutase (Breitman and Bradford, 1967, 1968; Hoffee and Robertson, 1969; Munch-Petersen, 1968a). In these mutants thymine is incorporated into DNA by the conversion of thymine and deoxyribose 1-phosphate to thymidine, a reaction catalyzed by thymidine phosphorylase. Uridine blocks this conversion if the level of thymidine phosphorylase is low, as in uninduced cells. Consequently the growth of low thymine-requiring cells should be inhibited on minimal glucose plates containing low thymine when uridine is added. Mutants deficient in deoxyribose 5-P aldolase are phenotypically constitutive for thymidine phosphorylase due to an endogenous accumulation of the inducer deoxyribose 5-phosphate (Barth *et al.*, 1968; Breitman and Bradford, 1968; Hoffee, 1968a) and their growth is not inhibited by uridine. Deoxyribose 5-P aldolase mutants, therefore,

cannot be used in this selection procedure. Mutants deficient in PDR mutase, on the other hand, contain uninduced levels of thymidine phosphorylase, unless grown in the presence of deoxyribose, and are inhibited from growing on minimal glucose plates containing low thymine and uridine. PDR mutase mutants can overcome this inhibition by uridine if they develop a mutation in their regulation which makes them constitutive for thymidine phosphorylase. Such mutants are described here.

Studies on these regulatory mutants have lead to the following observations:

(1) Most spontaneous mutants that have been isolated as constitutive for thymidine phosphorylase are also constitutive for the other three enzymes of the *deo* regulon.

(2) The gene for constitutivity described here shows no linkage to the *deo* genes in P22 transduction studies. It is therefore not characteristic of an operator type mutation.

(3) Mutants have been isolated which are temperature sensitive constitutives suggesting that the regulatory gene involved codes for a protein product.

The existence of strains constitutive for some of the *deo* enzymes has been reported previously (Lomax and Greenberg, 1968; Munch-Petersen, 1968a). Munch-Petersen, working with thymine-requiring strains of *E. coli* obtained from Okada (1966), reported the finding of a mutant Y70-22 which had constitutive levels of deoxyribose 5-P aldolase and thymidine phosphorylase. This strain contained a defect in PDR mutase and was thought to be the result of a deletion encompassing part of the *deo B* gene and the operator for the *deo A—deo C* genes (Ahmad and Pritchard, 1969). Recently, Ahmad and Pritchard (1971) have shown this strain to be a double mutant possessing a defect in the *deo B* gene and one in a regulatory gene not linked to *deo B* by P_1 transduction. This *nuc R* gene appears to map in *E. coli* near *gal E* (Ahmad and Pritchard, 1971). The defect in the regulatory gene is responsible for Y70-22 possessing high levels of three of the enzymes of the operons: deoxyribose 5-P aldolase, thymidine phosphorylase and purine nucleoside phosphorylase (Ahmad and Pritchard, 1971; Munch-Petersen, 1968a).

It would seem from the available data that the *deo* regulon is made up of four structural genes composing at least two closely linked operons *deo D—deo B* and *deo A—deo C*. These two operons appear to be under the control of a single regulatory gene (Ahmad and Pritchard, 1971; Robertson *et al.*, 1970). The product of the regulatory gene, presumably a protein, can interact with deoxyribose 5-phosphate, resulting in transcription of all four of the *deo* genes or it can interact with purine ribonucleosides resulting in transcription of only *deo B* and *deo D*. The regulatory gene does not appear to be closely linked to the *deo* enzymes. Studies are now in progress to determine the map position of the *deo R* gene, to isolate super suppressed (*deo* R^s) strains and operator constitutive mutants.

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Dr. James Blank
Dr. Patricia Hoffee
Department of Microbiology, School of Medicine
University of Pittsburgh
Pittsburgh, Pennsylvania 15213, U.S.A.