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Experimentation to Develop Procedures to be used in the Investigation of the Effects of Restriction on the Unmodified DNA of the Bacteriophage Lamda

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Submitted to
The Honors College,
Western Michigan University
June 5, 1973

The results of experimentation approximately twenty years ago with various bacteriophages by Ralston and Krueger[16], Anderson and Felix[1], Luria and Human[12], and by Bertani and Weigle[2] have shown that the host range of a given phage depends directly on the bacterial strain on which the phage has last propagated. This event came to be known as host-induced modification or host-controlled variation. As this phage host range is controlled by the host environment and can be altered from one generation to another it has, according to Luria, made microbiology "the last stronghold of Lamarckism".[11]

Host-controlled variation was one of the first qualities of bacteriophage λ to be studied. When λ is grown on its natural host, Escherichia coli K12 (Lederberg and Lederberg)[9], it is known as λ.K. E. coli C is also a host for phage λ and produces phage that are labeled λ.C. It was observed (Bertani and Wiegle)[2] that λ.C grew on E. coli K12 with a low efficiency of plating, approximately 2×10^{-4} yet grew well on E. coli C. Lambda.C are said to be restricted on E. coli K12. On the other hand, λ.K grew well on both E. coli K12 and E. coli C. Thus, while one bacterial strain may possess one or more modification-restriction mechanisms, some do not possess any, as in the case of E. coli C, and are designated type 0 strains.

Other initial work done in this area has shown that restriction acts at the DNA level. Dussoix and Arber[4] infected E. coli K12 lysogenic for phage P1 with phage λ.K and found that within minutes, more than half of the unmodified λ phage DNA, labeled with ^{32}P, appeared in acid soluble form.

Such breakdown of unmodified DNA necessitated the presence of specific nucleases in the restrictive bacterial strains. A number of investigators have detected such enzymatic activity in bacterial extracts.

Meselson and Yuan[13] used E. coli K12 as the enzyme source and phage λ DNA as a substrate. They incubated the bacterial extract with ^{32}P-labeled unmodified λ.C DNA and ^{3}H-labeled modified λ.K DNA and then determined the sedimentation pattern of the DNA in a sucrose gradient. The sedimentation velocity of the modified λ.K DNA remained unchanged while that of the unmodified λ.C DNA was considerably reduced. It was also found that both DNA's underwent breakdown upon ten minutes of incubation with purified restriction enzyme. Even prolonged incubation, however, did not cause extensive breakdown and indicated that restriction is due to an endonuclease acting at a limited number of sites on unmodified DNA. The more extensive degradation observed in vivo being the result of supplemental secondary action by exonucleases following the initial breaks by the endonuclease.

E. coli K-specific restriction requires ATP, Mg^{++}, and S-adenosylmethionine (SAM) as cofactors, although their roles are not clearly understood. It is speculated that SAM acts as a methyl donor in restrictive enzymatic methylation of DNA. It is known, however, that methylation of purine residues, particularly at the N7 position of guanine or the N3 position of adenine, leads to depurination of a polydeoxyribonucleotide. The K12 specific endonucleases responsible for restriction is expected to catalyze the methylation of certain purine residues (proposed by L. Linn and J. P. Brockes[10]) and would be followed by local depurination, making

the sugar phosphate linkage prone to hydrolysis.

Endonuclease K is inactivated in less than two minutes in the presence of Mg^{++}, ATP, S-adenosylmethionine, and modified DNA. Degradation of unmodified DNA, however, takes considerably longer. It would seem the enzyme is protected from inactivation by binding to unmodified DNA. Binding studies show that endonuclease K interacts strongly and specifically with unmodified DNA.

Modification, the result of DNA methylation, prevents what would amount to cellular suicide, by protecting intracellular DNA from degradation by the restriction endonuclease. Methylation by modification methylases occurs at or near the endonuclease cleavage sites on the polynucleotide chains. Modification differs from restriction in that Mg^{++} and ATP are not required, but they are similar in that SAM is a necessary cofactor. S-Adenosylmethionine is the proven methyl donor of the reaction. By using ^{3}H-labeled methyl groups of SAM, it has been shown that the methylation site on the unmodified DNA is the extracyclic amino nitrogen of adenine.[8]

Methylase activity has been noted in endonuclease K when incubated with unmodified λ DNA and SAM. In addition, the resulting methylated DNA is resistant to endonucleolytic cleavage in the presence of the endonuclease, Mg^{++}, ATP, and SAM. Such methylase activity by the restriction enzyme is not consistent in other restriction systems. Nevertheless, investigators posit the existence of a complex enzyme molecule composed of subunits able to dissociate and split off a functional modification or restriction enzyme. It has been hypothesized that the K modification-restriction system is controlled by three genes.[3,7] They have been designated hsr, hss, and hsm. According to this model, the product of hsr is essential to restriction, the

product of hss essential to the recognition of the specific system, and the product of hsm essential to modification. Both restriction and modification enzymes apparently contain the hss gene product. Enzyme subunits have been separated having molecular weights of 136,000, 60,000, and 55,000 and are thought to be products of hsr, hsm, and hss respectively.

Recent studies of DNA have employed the specificities of different restriction enzymes. By cleaving the DNA at their respective base sequences and producing overlapping DNA fragments, nucleotide sequencing is being facilitated.[14]

Preliminary results by J. Schell, M. Zabeau, J. Heip, and M. Van Montagu (personal communications) indicate that cleavage of the unmodified DNA is not the sole inhibitory mechanism of restriction; but that it prevents the transcription of restricted DNA as well. It was the purpose of this paper to develop an assay to confirm or refute this possibility.

The amino acid tryptophan is synthesized via a pathway that includes the catalysis, by the enzyme anthranilate synthetase, of chorismic acid to anthranilic acid. A strain of phage λ carrying the tryptophan (trp^+) genes has been constructed by Franklin.[5]

If restriction prevents transcription, infection of a trp E^- mutant <u>E. coli</u> K12 (one that cannot synthesize anthranilate synthetase) with a λ trp^+.0 phage (unmodified), should not result in even low levels of anthranilate synthetase production.

Conversely, a λ trp^+.K phage grown on the <u>E. coli</u> K12 trp E^- mutant should begin transcribing for tryptophan. The same results are expected if the λ trp^+.K phage is re-infected on its trp E^- parent.

This paper deals primarily with the isolation of the necessary mutants as well as the testing and evaluation of the necessary enzyme assay system.

Materials and Methods

Organisms:

Five E. coli K bacteria were used throughout this study. They are listed below followed by their genotypes:

1) PAZ-35; B_1^-, arg A^-, trp E^-, Sm^R, F^-, xyl^-, mal^-, mtl^-
2) C3000; su^-, HfrH, thi^-
3) CR63; su^+
4) Kr^-m^-
5) 159; Uvr^-, gal^-, su^-

In addition, mutants were selected from PAZ-35 and C3000.

The bacteriophage utilized was a lambda hybrid having a λ head and h^{80} tail fibers[5]:

λ trp 46; h^{80}, trp D^+E^+, N^+, i, λ CIAt2

Phage Modification and Harvest:

The K modification was conferred upon the λ phage (λ trp^+.K) by growing it on CR63. Likewise the λ trp^+.O (unmodified) phage was obtained by harvesting phage grown on Kr^-m^-. The harvesting procedure for both the modified and unmodified phage follows:

Cultures of CR63 and Kr^-m^- were grown overnight in a broth containing (grams per liter): tryptone, 10.0; NaCl, 5.0; and $MgSO_4.7H_2O$, 0.246. One-tenth milliliter phage stock, diluted to 1×10^6 phage/ml, was plated with o.3 ml of the respective bacteria cultures and 3.0 ml of soft agar (gram per liter: tryptone 10.0; NaCl, 8.0; and agar, ~~15.0~~ 6.0). on an agar base.

The plates were incubated 6-8 hours at 37 C. until confluent lysis occurred. The soft agar layer was scraped off the plates. To it was added 10 ml of λ buffer, pH 7.2 (0.01 M tris(hydroxymethyl) amino methane (Tris) hydrochloride, and 0.01 M $MgSO_4$) containing 1% chloroform

and the mixture was shaken well. It was then centrifuged 10 minutes at 10,000 rpm and the supernatant stored without chloroform at 5 C.

Selection of thymine minus mutant:

The procedure for thy^- selection using trimethoprim, was similar to that described by Miller.[15] Trimethoprim (2,3-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine) depresses the growth of thy^+ cells, but not that of thy^- cells in the presence of concentrations of thymine of at least 50 ug/ml. E. coli PAZ-35 was subcultured overnight in nutrient broth. A drop of the culture was plated on trimethoprim agar plates containing (g/l): basal salts ($MgSO_4.7H_2O$, 0.3; citric acid.H_2O, 3.0; $K_2HPO_4.4H_2O$, 5.2); agar, 15.0; D-glucose , 4.0; and also containing (ug/ml): thiamine, 0.5; L-tryptophan, 80.0; L-arginine, 80.0; thymine, 50.0; and trimethoprim, 10.0. The plates were incubated at 37 C. Any colonies arising from these plates were carefully picked and resuspended in sterile saline (NaCl, 8.5 g/l). They were then streaked onto trimethoprim agar and onto plates minus both trimethoprim and:

1) tryptophan
2) arginine
3) thymine

Finally, the cells were plated onto agar minus trimethoprim but containing tryptophan, arginine, and thymine. As the PAZ-35 strain was was originally arg^- and trp^-, true PAZ-35 thy^- mutants should not grow on agar plates 1, 2, or 3 described above, but should grow on plates minus trimethoprim but including thy, arg, and trp. This plating insured the mutant selected was from the original PAZ-35 strain.

Mutagenesis and isolation of trp E^- mutants:

The mutagenesis and trp E^- selection procedure was similar to that

described by Miller[15]. E. coli C3000 was subcultured overnight in Luria broth (g/l: tryptone, 10.0; yeast extract, 5.0; and NaCl, 0.5) at 37 C. The cells were centrifuged and washed twice the next day with sterile 0.1 M citrate buffer (pH 5.5) and resuspended in 4.0 ml of the same buffer. N-Methyl-N'-nitro-N-nitrosoguanidine was added to a final concentration of 50 ug/ml and the cells were allowed to stand for 25 minutes in a 37 C. water bath. Nitrosoguanidine treatment of C3000 at 50 ug/ml for 25 minutes results in approximately 50% killing as indicated from a previously determined killing curve.

After 25 minutes, the treated cells were centrifuged and washed once in sterile 0.1 M sodium phosphate buffer (pH 7.0). They were resuspended in minimal medium A containing (g/l): K_2HPO_4, 10.5; KH_2PO_4, 4.5; $(NH_4)_2PO_4$, 1.0; sodium citrate.$2H_2$), 0.5; D-glucose, 4.0; $MgSO_4$, 0.12; plus (ug/ml) tryptophan, 100; and thiamine, 5.0. The cells were incubated overnight at 37 C in a New Brunswick incubator-shaker.

On the third day, the cells were centrifuged and washed twice with sterile sodium phosphate buffer, innoculated to a density of 1-2 x 10^7 cells/ml in a side arm flask containing minimal medium A minus tryptophan, and grown at 37 C. in the incubator-shaker. The initial optical density was measured using a Klett-Summerson colorimeter with a 550 nm filter. The cells were incubated till the optical density increased four to five times. At this point, penicillin G (1590 units/mg) was added to the culture to a final concentration of 20,000 units/ml, lysing the growing trp^+ cells yet leaving the dormant trp^- cells intact. This mixture was incubated an additional 60 minutes during which time a measurable decrease in optical density, due to lysis, was observed using the colorimeter.

The cells were centrifuged, washed with sterile sodium phosphate

buffer and resuspended in minimal medium A plus tryptophan and again incubated overnight at 37 C.

The procedure of day three was repeated on day four.

On day five, 1×10^{-6} dilutions from the culture were plated on glucose minimal plates plus tryptophan and replicated on glucose minimal plates plus indole (100 ug/ml), on glucose minimal plates, and again on glucose minimal plates plus tryptophan. Tryptophan E^- mutants should grow when replicated on glucose minimal plates containing indole or tryptophan, but not on unadulterated glucose minimal plates.

Enzyme assay:

The synthetase assay was essentially that described by Zalkin and Kling.[19] It is based on the fact that anthranilic acid fluoresces at 400 nm when exposed to an excitation light of 325 nm. Given a mixture of chorismic acid substrate and the synthetase enzyme, the amount of flourescence, i.e. the amount of anthranilic acid formed can be measured.

The assay was tested with bacterial extracts prepared from PAZ-35 (trp E^-) and wild type 159 (trp E^+). The cells were harvested at a density of $4\text{-}5 \times 10^8$ cells/ml from media composed of (g/l): acid-hydrolyzed, vitamin-free Casamino Acids, 5.0; basal salts ($mgSO_4 . 7H_2O$, 0.3; citric acid.H_2O, 3.0; $K_2HPO_4 . 4H_2O$, 5.2); and D-glucose, 4.0 (60 ug/ml L-tryptophan was also added to the PAZ-35 medium). The cultures were centrifuged and washed once in a mixture of basal salts and casamino acids. The cells were centrifuged again, resuspended in Tris buffer, and disrupted with a Branson model S-125 sonifier using three, 15 second bursts. The sonicated mixtures were centrifuged at 35,000 x g for 15 minutes. The resultant supernatant fluids were retained for synthetase analysis. All procedures were carried out at 4 C.

The assay mixture was composed of 0.2 ml enzyme, 0.2 ml chorismic

acid substrate (o.3mM), 0.2 ml thioglycerol (2.0 mM), and 0.2 ml of a buffer (pH 8.5) that included $(NH_4)_2SO_4$ (50 mM), triethanolamine hydrochloride (50mM) and MgCl (10 mM). Relative fluorescence was measured using an Aminco-Bowman spectrophotofluorometer. Precautionary runs were made with 0.2 ml water substituted for substrate to make certain the enzyme harbored no endogenous fluorescent activity.

The amount of anthranilic acid formed was calculated from a previously determined standard curve of anthranilic acid fluorescence.

Protein determination:

Protein was determined (Table 1) using a method described by Lowry.

Results

Isolation of thy^- and $trp\ E^-$ mutants:

To eliminate any extraneous effects caused by viral DNA replication, it was necessary to select for a thy^- host to prohibit such activity.

The selection for PAZ-35 thy^- mutants was very successful. Twenty-four out of twenty-five colonies selected were thy^-. These were checked for revertant mutations and two stable mutants were retained.

At this point the PAZ-35 thy^- mutant was infected with λ.K phage (modified to insure that it was a suitable host. No lysis, however, was observed from either mutant. Evidently neither PAZ strain possessed the h^{80} receptor sites.

The PAZ-35 thy^- mutant was put aside in favor of a susceptible host that did lyse when infected with λ.K. This host was C3000. But as C3000 was $trp\ E^+$, it was now necessary to introduce the $trp\ E^-$

mutation as well as the thy$^-$ mutation.

The first enrichment on tryptophan for the C3000 trp E$^-$ auxotroph yielded approximately 40-50 trp$^-$ mutants. Some of these were restreaked onto glucose minimal plates plus anthranilic acid to separate the trp E$^-$ mutants ffom the rest. No growth was observed on this substrate and the remaining trp$^-$ mutants were prematurely discarded.

At present, a second tryptophan enrichment has yielded four possible trp E$^-$ mutants (growing on both glucose minimal plates plus tryptophan or indole but not on unadulterated plates) and are being tested enzymatically for loss of anthranilate synthetase. One of these has proven to be trp E$^-$.

Assay development:

The data from Table 1 clearly shows the synthetase activity of the 159 trp E$^+$ extract. On the other hand, no activity was detected from the PAZ-35 trp E$^-$ extract.

Table 1

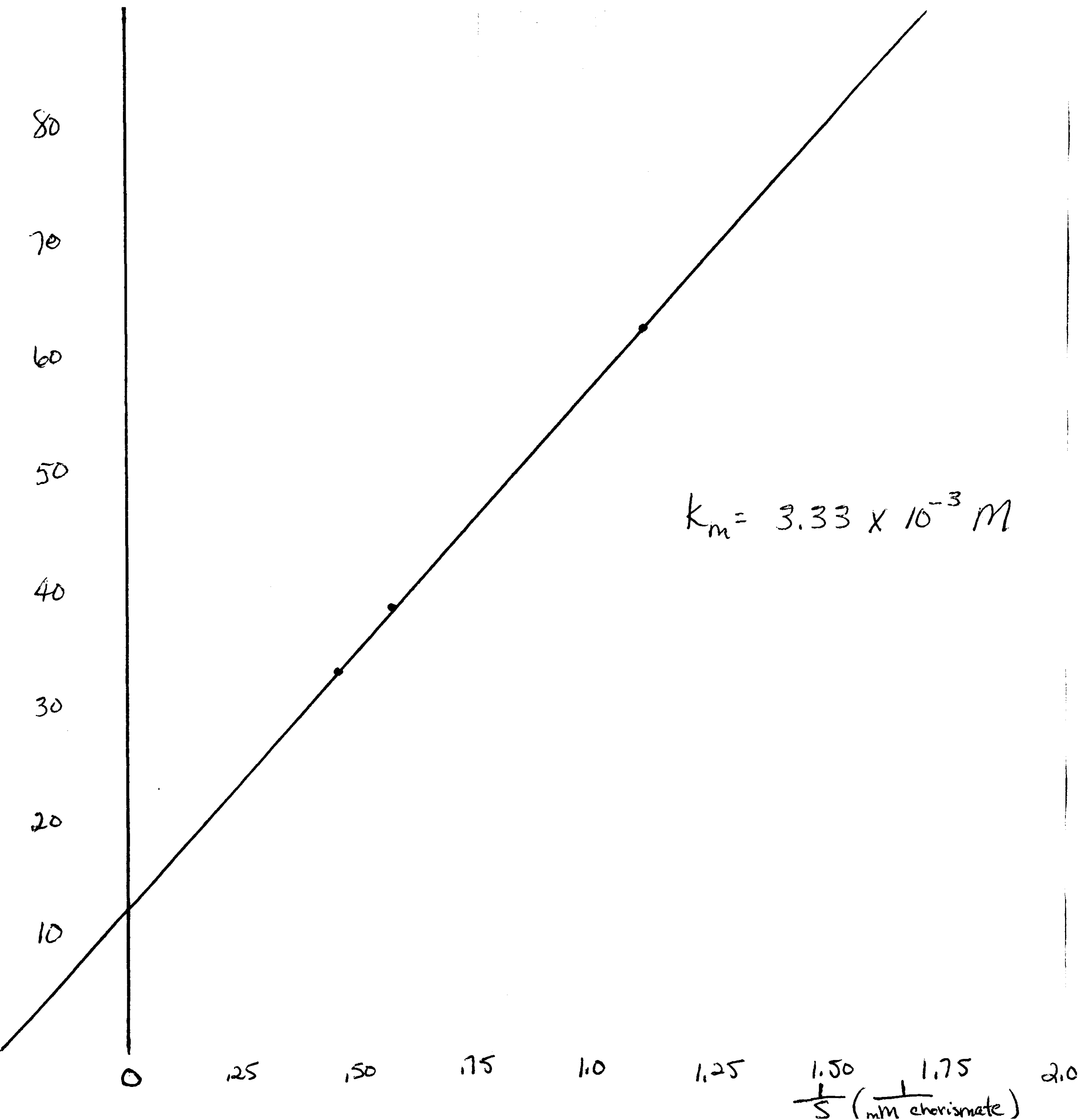
Strain	Anthranilate synthetase specific activity[a]
159 (wild type)	165
PAZ-35 trp E$^-$	0

([a]Micromoles of anthranilic acid formed per minute per milligram protein.)

This data **also** confirms that the substrate synthesized in the lab (Gibson and Gibson[6]) was indeed chorismic acid as activity was observed from the trp E$^+$ strain and not from the trp E$^-$ strain.

A Lineweaver-Burk plot of enzyme activity (Graph 1) agrees with Michaelis-Menten kinetics. The calculated K_m is 3.33×10^{-3} M chorismate.

Graph 1: Lineweaver-Burk plot of synthetase activity



Graph 1

Discussion:

The K_m value for chorismate disagrees with that published by Yanofsky and Ito[18] (3×10^{-5} M) for E. coli. They, however, used a different buffering system and at a lower pH of 7.8.

Working with the anthranilate synthetase of Salmonella typhimurium, but using a buffer system at a pH close to that described in the Materials and Methods, Tamir and Srinivasan[17] have calculated a K_m for chorismate equalling 5.0×10^{-3} M which is in better agreement with the K_m of this paper.

The experiment would have proceeded much more smoothly had the PAZ-35 thy$^-$ mutant proved to be a suitable host for λ trp$^+$.K. As it was, difficulties were encountered while selecting for the C3000 trp E$^-$ mutant. Even more time would have been saved had the cells grown on onthranilic acid. It would seem that C3000 does not transport anthranilic acid. Enzymatic testing must now be performed on the potential trp E$^-$ mutants to determine if they have lost synthetase activity.

Once the C3000 trp E$^-$ mutant has been successfully isolated, it is anticipated that productive experimentation will shortly follow; due to the reliability of the trimethoprim thy$^-$ selection and the proven feasibility of the synthetase assay.

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