

Degradation of Pyrimidines by *Pseudomonas Syringae*

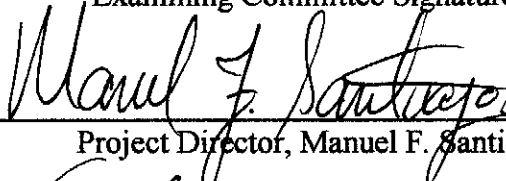
by
Randi L. Gant

Departmental Honors Thesis
The University of Tennessee at Chattanooga
Department of Chemistry

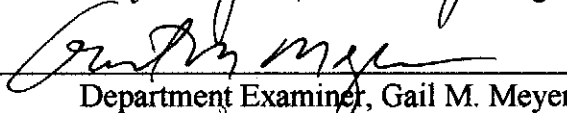
Project Director: Manuel F. Santiago
Examination Date: March 23, 2005

H. Douglas Kutz
Gail M. Meyer
David Sachsman

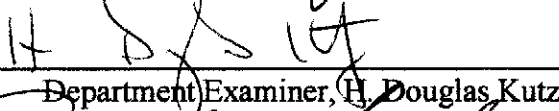
Examining Committee Signatures:



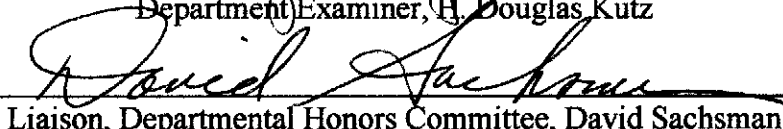
Project Director, Manuel F. Santiago



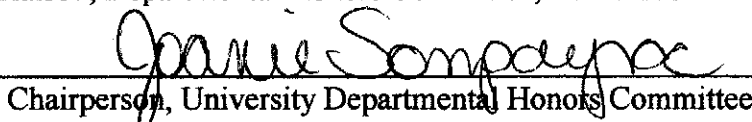
Department Examiner, Gail M. Meyer



Department Examiner, H. Douglas Kutz



Liaison, Departmental Honors Committee, David Sachsman



Chairperson, University Departmental Honors Committee

Table of Contents

Table of Figures	5
Introduction.....	6
Materials and Methods	16
Results	22
Discussion.....	66
References.....	69
Acknowledgements	71

List of Tables

	Page
1. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source.....	23
2. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with no supplemental nitrogen source.....	25
3. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with thymine as a nitrogen source.....	26
4. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrouracil as a nitrogen source.....	28
5. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrothymine as a nitrogen source.....	29
6. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-alanine as a nitrogen source.....	30
7. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-aminoisobutyric acid as a nitrogen source.....	31
8. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with ammonium sulfate as nitrogen source	32
9. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with cytosine as a nitrogen source	33
10. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with no supplemental nitrogen source.....	34
11. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with thymine as a nitrogen source.....	36
12. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with uracil as a nitrogen source.....	37
13. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with dihydrouracil as a nitrogen source.....	38
14. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source	

with dihydrothymine as a nitrogen source.....	39
15. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with Beta-alanine as a nitrogen source.....	40
16. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with Beta-aminoisobutyric acid as a nitrogen source.....	41
17. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with ammonium sulfate as a nitrogen source.....	42
18. Pyrimidine reductive pathway enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with cytosine as a nitrogen source	43
19. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with no supplemental source of nitrogen and either NADH or NADPH as a reducing cofactor.....	46
20. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor	47
21. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with thymine as a nitrogen source and either NADH or NADPH as a reducing cofactor	48
22. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrouracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	49
23. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrothymine as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	50
24. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-alanine as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	51
25. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-aminoisobutyric acid as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	52
26. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with ammonium sulfate as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	53

27. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with cytosine as a nitrogen source and either NADH or NADPH as a reducing cofactor	54
28. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with no supplemental source of nitrogen and either NADH or NADPH as a reducing cofactor.....	56
29. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor	57
30. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with thymine as a nitrogen source and either NADH or NADPH as a reducing cofactor	58
31. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with dihydrouracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	59
32. Dihydropyrimidine Dehydrogenase enzyme activities for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing succinate as the carbon source with ammonium sulfate as a nitrogen source and either NADH or NADPH as a reducing cofactor.....	60
33. Pyridine Nucleotide Transhydrogenase specific activity for <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium.....	62
34. Pyrimidine reductive pathway enzyme activities for penicillin resistant <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source	64
35. Dihydropyrimidine Dehydrogenase enzyme activities for penicillin resistant <i>Pseudomonas syringae</i> ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.	65

Table of Figures

1. Pyrimidine Base Structure	8
2. Uracil, Thymine, and Cytosine.....	10
3. The Uracil Reductive Pathway.....	12
4. The Thymine Reductive Pathway	13

Introduction

Pseudomonas syringae ATCC 12771 is a bacterial plant pathogen that is responsible for diseases that affect a wide assortment of plants, including rice, tobacco, legumes, tomatoes, and oats (all of which are important agricultural products). Because significant amounts of crops are lost annually to diseases caused by this organism, there is growing interest in regulating its metabolic process. Currently techniques for controlling the growth of this organism are limited; very little has been investigated to understand this organism's biochemistry to control its growth without negatively influencing the ecosystem. Pesticides, which are the current dominant method of controlling the *P. syringae*, devastate the ecosystem that is exposed to it by not only killing the pathogen, but also the entire ecological community of organisms similar to it (Naidu, et al. 1998). The pesticides also build up in the food chain by accumulating in the tissues of organisms that consume the exposed microorganisms. One such well known example is the build up of Dichlorodiphenyltrichloroethane, or DDT, a mosquito pesticide used in the 1930's, in the tissues and egg shells of bald eagles and thereby contributing to the bird's endangerment (Naidu, et al 1998).

The use of biochemical control agents is gaining in popularity as an alternative to pesticides because of increased environmental awareness and knowledge of food and water cycles. Biochemical control agents are substances that regulate or inhibit the growth of specific organisms or groups of organisms without negatively impacting other members of the ecosystem. In order to develop and utilize biochemical control agents, the biochemistry of the organism must be understood.

Biosynthesis and degradation of biomolecules are two biochemical processes which contribute to an organism's metabolism and growth. Inadequate nutrient supply, environmental conditions, or even space can inhibit an organism's ability to undergo either of these processes.

Two essential components of biomolecules for an organism are carbon and nitrogen. These two elements are found in nearly every biochemical process in a cell. They are used to biosynthetically develop organelles, deoxyribonucleic acid (DNA), proteins, and other cellular structures. Pyrimidines are one such example of biomolecules formed from carbon and nitrogen (Figure 1). These are aromatic rings that are found in numerous places inside the cell, most notably as components of nucleic acids, which are used to synthesize DNA and enzymatic proteins, that assist in performing the metabolic functions of the cell.

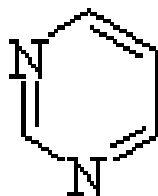


Figure 1: Pyrimidine Base Structure

When an organism is placed in a nitrogen-free environment and starved of nitrogen, it will begin to express genes through transcription that will allow the cell to degrade excess nitrogen-containing molecules within the cell. It is this catabolism of nitrogen-containing molecules (most importantly from amino acids and proteins) that provides nitrogen for the biosynthesis of other important molecules for growth. Pyrimidine degradation is one such method that releases carbon and nitrogen from its aromatic rings for the purpose of biosynthesis and growth.

There are three pyrimidines of interest for this investigation: uracil, thymine and cytosine (Figure 2). There are two primary mechanisms for pyrimidine degradation: the oxidative pathway and the reductive pathway (O'Donovan, et al 1970). The oxidative pathway is utilized in a relatively small number of organisms, and it is a two step process to catabolize the pyrimidines. In the Uracil Oxidative Pathway, the first step is catalyzed by the enzyme uracil dehydrogenase (EC 1.2.99.1) to oxidize uracil to barbituric acid. The second step is catalyzed by the enzyme barbiturase (EC 3.5.2.1) to convert barbituric acid to urea and malonic acid. The process is similar for the thymine and cytosine Oxidative pathways.

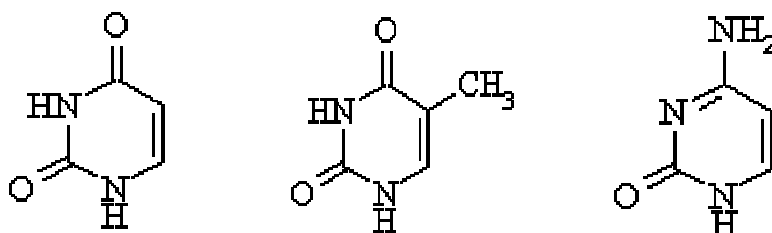


Figure 2: Uracil, Thymine, and Cytosine

The reductive pathway is a more prevalent pathway in bacteria (Wang, et al 1951). It is a three step process for the reduction of uracil. In the first step, the enzyme dihydropyrimidine dehydrogenase converts uracil to dihydrouracil. This essentially breaks the carbon-carbon double bond on the third and fourth carbons with nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine phosphate dinucleotide (NADPH) reduction. In the second step, the enzyme dihydropyrimidinase converts dihydrouracil to N-carbamoyl-Beta-alanine. This does so by breaking the bond along the number one carbon and adjacent nitrogen of the aromatic ring through hydrolysis. The product is stabilized by oxidizing the first carbon with an alcohol substituent. In the third step, the enzyme N-carbamoyl-Beta-alanine amidohydrolase converts N-carbamoyl-Beta-alanine to Beta-alanine. This does so by hydrolysis of the nitrogen bond between the fourth position from the carbonyl group and the fifth position to form carbon dioxide, ammonia, and Beta-alanine (Figure 3). A similar pathway can be found for thymine (Figure 4). Cytosine is converted to uracil in the cytosine deaminase reaction and from there undergoes the uracil reductive pathway (Vogels, et al 1976).

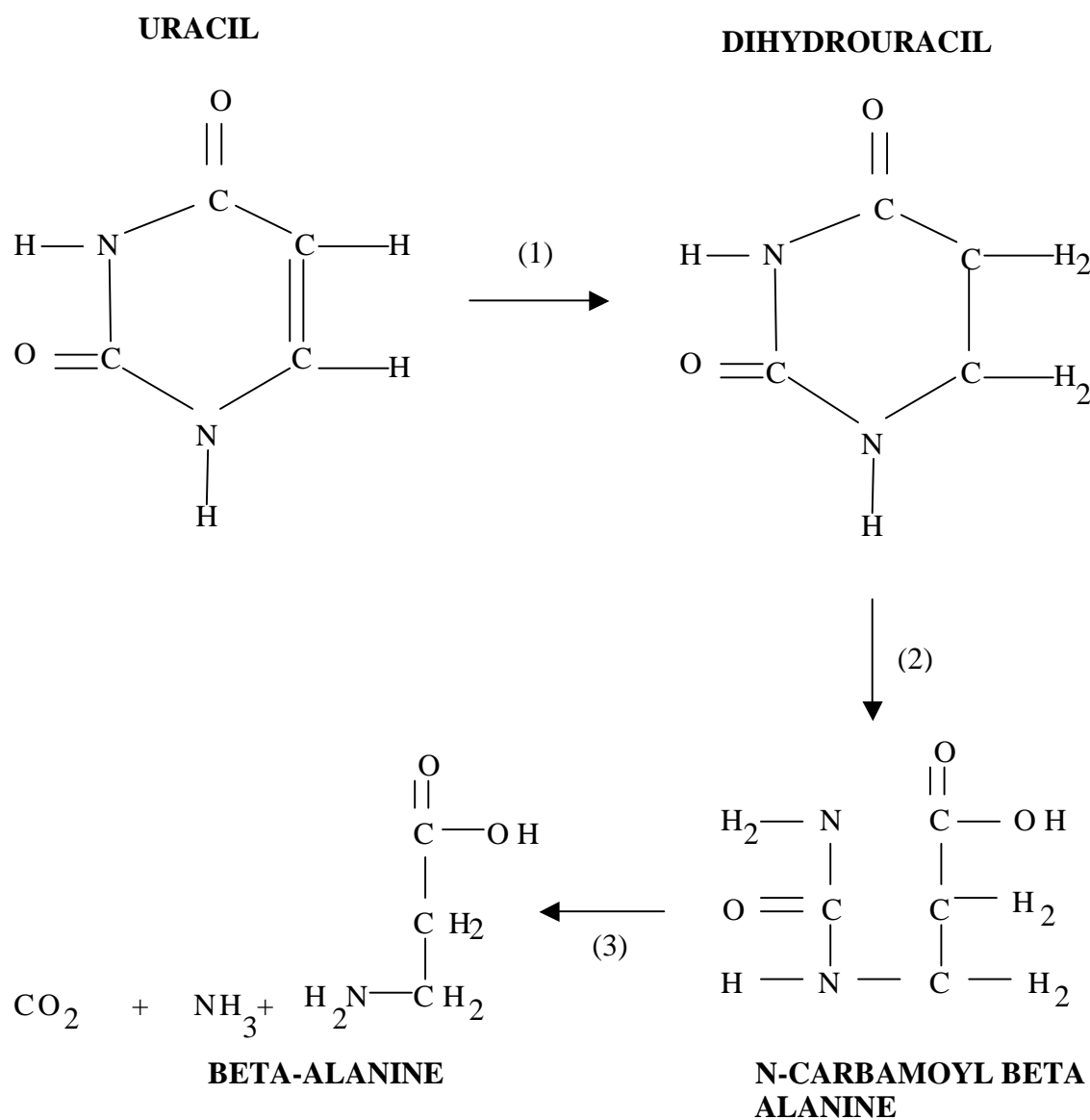


Figure 3: The Uracil Reductive Pathway enzymes: 1) Dihydropyrimidine Dehydrogenase, 2) Dihydropyrimidinase, 3) N-Carbamoyl-Beta-Alanine Amidohydrolase.

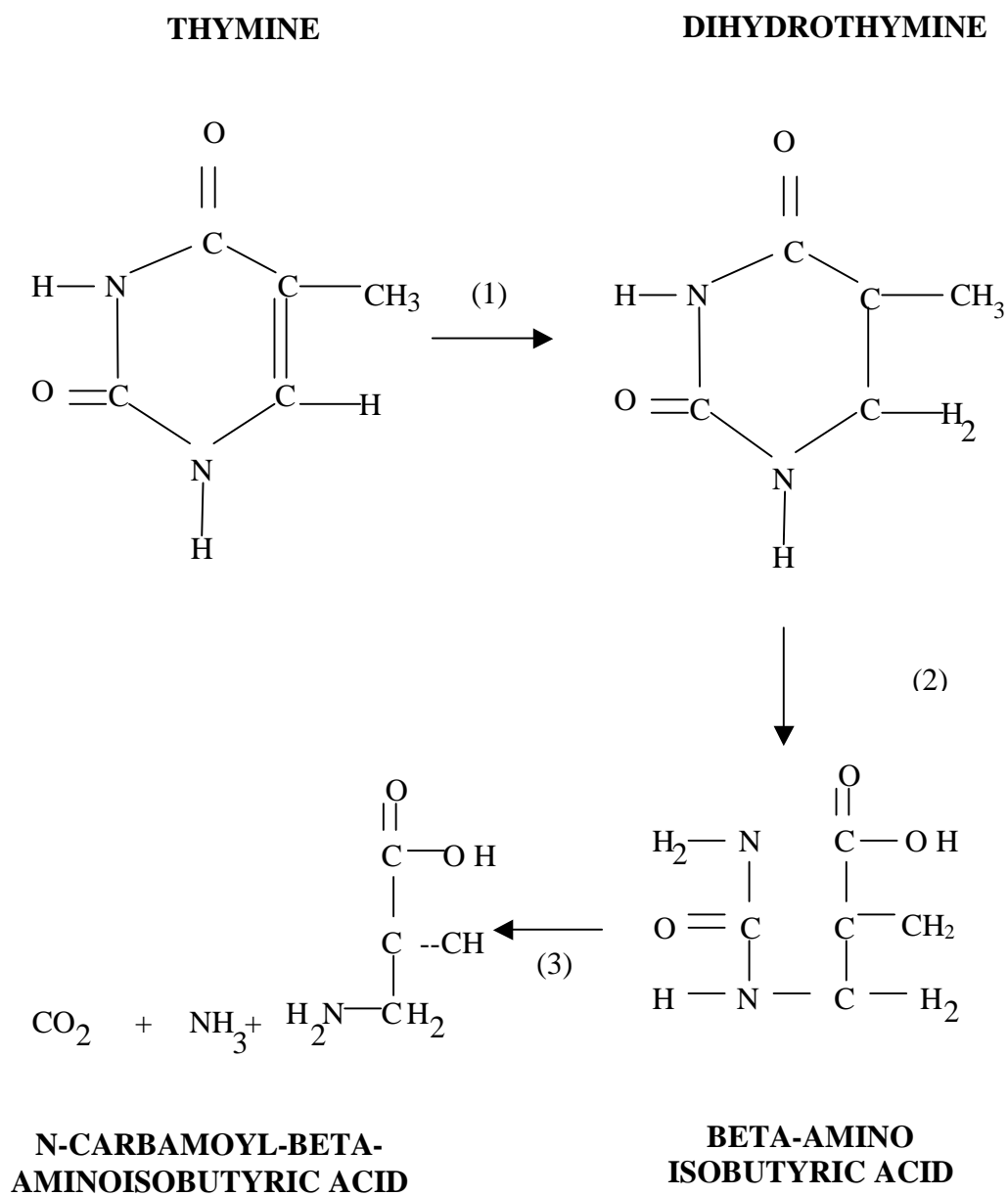


Figure 4: The Thymine Reductive Pathway enzymes: 1) Dihydropyrimidine Dehydrogenase, 2) Dihydropyrimidinase, 3) N-Carbamoyl-Beta-Aminoisobutyric Acid Amidohydrolase.

It has been shown in other members of the genus *Pseudomonas* (such as *Pseudomonas stutzeri*, *Pseudomonas fluorescens*, and *Pseudomonas lemmonieri*) that the pyrimidine reductive pathway is the primary mechanism for the degradation of thymine, uracil, and cytosine (West, et al. 1982, 1989; Xu and West, 1992; Santiago and West, 1999). It has also been shown in these organisms that the pathway for this degradation utilizes the three enzymes: dihydropyrimidine dehydrogenase, dihydropyrimidinase, and N-Carbamoyl- β -Alanine amidohydrolase. It is believed a great deal of taxonomic identification information can be obtained by comparing the catabolic pathway of *P. syringae* with what is known of the catabolic pathways of other members of its genus.

It is the intent of this project to elucidate the chemical and enzymatic mechanisms of a portion of *P. syringae*'s biochemistry, namely the degradative pyrimidine biochemistry. This will have further application toward biochemical control agents. Specific activities of the enzymes of the reductive pathway will be assayed to determine the level of expression of the pathway; also, comparative enzyme assays of organisms grown on different nutrient sources will provide control groups for determination of the specific activities. By exposing the organism to various nutrient sources, the study should reveal whether the pathway can be biochemically regulated, thus controlling its ability to damage cash crops.

As implied before, this project is also undertaken to investigate the pathway in *P. syringae* cells for comparison with the other members within this genus. The results from this investigation can also be used to help in the taxonomic assignment of this organism. The reducing cofactor of the dihydropyrimidinase dehydrogenase

reaction, for instance, could reveal differences between this organism and other members of the genus. Typically, bacteria (prokaryotes) use NADH as a cofactor for the reaction, while eukaryotes typically use NADPH for the reaction. By performing the first assay using either NADH and NADPH and comparing the enzyme activities, it will be revealed which is the prevalent cofactor for the reaction in this organism.

Antibiotic-resistant bacteria are increasingly becoming a concern in medicine and agriculture. These bacteria become immune to the effects of antibiotics by genetically mutating in order to survive, and are more difficult to eliminate and even control due to their genetic mutations. Because antibiotic-resistant bacteria are increasingly becoming a problem in controlling infections and plant blight, there is a need to understand how the genetic mutations affect the organisms expression of enzymes. This project will investigate the connection between antibiotic mutagenesis and changes in enzyme specific activity of the reductive pathway.

Materials and Methods

Chemicals

Uracil, dihydrouracil, thymine, dihydrothymine, Beta-alanine, Beta-aminoisobutyric acid, cytosine, ammonium sulfate, sodium succinate, glucose, potassium bicarbonate, penicillin, Spectra/Por Regenerated Cellulose Membrane (dialysis tubing), mercaptoethanol (dialysis solution), Coomassie brilliant blue, Hydroxymethyl aminomethane (TRIS), hydrochloric acid, ethylene diamine tetraacetic acid (EDTA), lysozyme, glutamate dehydrogenase, reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), antiprine, agar, nutrient broth, iron (III) chloride, glutamic acid, magnesium sulfate heptahydrate, ethanol 200 proof, phosphoric acid. These chemicals were purchased from Acros and Sigma.

Microorganism and Growth Media

The microorganism *Pseudomonas syringae* ATCC 12771 was used in this study. It was initially starved for 16 hours of all carbon and nitrogen sources except for one of each to serve as a control for enzyme activity. It was grown initially in a modified Stanier minimal liquid medium, that contained KH_2PO_4 (0.1% w/v), K_2HPO_4 (0.1% w/v), NaCl (0.1% w/v), sodium citrate (0.05% w/v), a carbon source of either glucose or succinate (0.4% w/v), and a nitrogen source (0.2% w/v) (Stanier, 1947) and was supplemented with iron chloride (0.02% w/v) and glutamic acid (10% w/v), which were found to be the optimal growth conditions (West, et al 1992).

Samples of eight different nitrogen sources were assayed independently with glucose or succinate as the carbon sources. These sources were uracil, dihydrouracil, thymine, dihydrothymine, beta-alanine, beta-aminoisobutyric acid, cytosine, and ammonium sulfate and one sample without nitrogen. The penicillin-resistant mutants were grown in liquid medium containing uracil or thymine as the nitrogen sources with glucose or succinate as the carbon source.

The cells were starved of the nitrogen and carbon sources for preparation for enzyme assays by transferring the cells from a nutrient agar plate to three flasks of fifty milliliter samples of liquid minimal medium with a single carbon and nitrogen source, shaken at 200 rotations per minute, and incubated at 30 degrees Celsius. The organisms were grown overnight. Two milliliters of these solutions were then reinoculated into three fresh flasks of fifty milliliters of liquid minimal medium to assure mid-exponential phase growth, and the growth was measured spectrophotometrically over periods of 1 and 2 hours at 600 nm. The generation time was determined by the time required for the population to double.

Preparation of Cell Extracts

At an absorbance of 0.5 to 0.7 (mid-exponential phase), the cells were washed once with sterile sodium chloride (0.85%), once with sterile deionized water, and collected by centrifugation at $7,719 \times g$ for 20 minutes at 4 degrees Celsius. The resulting cell pellet was resuspended in four milliliters of dialysis buffer. The dialysis buffer contained 20mM Tris-HCl buffer pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA), and 1mM Beta-mercaptoethanol (West, et al 1985). The cell extract was sonicated at maximum power in thirty second intervals for five minutes per

sample to lyse the cell membranes and liberate the proteins from the cell. The cell extracts were then centrifuged to remove the cell biomass from the solution. The resulting cell-free extract (containing the cell's enzymes) was then placed in dialysis tubing (Spectra/Por Regenerated Cellulose Membrane) in a 300 milliliter solution of 20mM Tris-HCl buffer (pH 7.5), 1mM EDTA and 1mM 2-mercaptoethanol at 4 degrees Celsius and allowed to dialyze overnight.

Enzyme Assays

The three reductive enzymes of the reductive pathway were assayed to determine the specific activities. Each enzyme had three independent culture samples that were analyzed, and from these a mean and standard deviation were calculated.

First Enzyme Assay: dihydropyrimidine dehydrogenase

The initial enzyme in the pyrimidine reductive pathway, dihydropyrimidine dehydrogenase, was assayed in a reaction mixture (1 mL total volume) containing 0.1 mM Tris-HCl buffer (pH 7.5), 0.2mM NADH or NADPH and cell-free extract (French, et al 1997). The mixture was incubated for five minutes at 30 degrees Celsius, and then 0.2 mL of either uracil or thymine (5 mM concentration) was placed in the reaction mixture as starting material. The control mixture contained deionized water (0.1mL). The conversion of NADPH to NADP⁺ or NADH to NAD⁺ was followed spectrophotometrically for 9.5 minutes at a wavelength of 340 nm. The conversion of the cofactor was calculated using a molar absorptivity coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The specific activity of dihydropyrimidine dehydrogenase was reported in nanomoles of dihydrouracil formed/ minute/milligram of protein.

Second Enzyme Assay: dihydropyrimidinase

The second enzyme of the reductive pathway, dihydropyrimidinase, was assayed for activity colorimetrically. The reaction mixture (1 mL) contained 0.1 M Tris-HCL buffer (pH 7.5), 0.01 M MgCl₂, and cell-free extract (Chu, et al. 1990). Dihydrouracil (0.1 mM) was added as an enzyme substrate to start the reaction. Three trials of 0 minute, 10 minute, 20 minute, and 30 minute reaction times were assayed. The reaction was stopped by the addition of an acidic color mix reagent (1 mL) consisting of one part oxime and two parts antipyrine. (West, et al 1982). The oxime was prepared by dissolving (5% v/v) of diacetylmonoxide in acetic acid (Prescott, et al 1969). The antipyrine was prepared by dissolving five grams of solid antipyrine (50% reagent grade) in 85% H₃PO₄. After termination of the reaction, the tubes were incubated at 70 degrees celsius for 120 minutes and were placed on ice until absorbances could be collected of each sample. The absorbances were read at 466 nm and plotted against a standard curve of known concentrations versus absorbance of N-carbamoyl-Beta-alanine. This curve was also used to calculate an experimentally calculated absorption coefficient that varied with each assay. The specific activity was reported in nanomoles of N-carbamoyl-Beta-alanine produced/minute/milligram of protein.

Third Enzyme Assay: N-Carbamoyl-Beta-alanine amidohydrolase

The third enzyme of the reductive pathway, N-Carbamoyl-Beta-alanine amidohydrolase, was assayed for activity. Beta-Alanine, ammonia, and carbon dioxide are the products in this catabolic reaction. The enzyme assay was coupled with a glutamate dehydrogenase enzyme assay followed spectrophotometrically at

365 nm. The first reaction mix (1 mL total volume) contained 0.1 M Tris-HCl buffer (pH 7.5), 0.01 MgCl₂, and cell-free extract (Tamiki, et al 1987). Dihydrouracil (0.1 mM) was added as an enzyme substrate to start the reaction. Three trials of 0 minute, 10 minute, 20 minute, and 30 minute reaction times were assayed. The reaction was stopped by the addition of 0.1 mL of 50% (w/v) trichloroacetic acid. The reaction mixtures were then centrifuged at low speeds, removing biomass not associated with the reaction of interest. The ammonium ion concentration in the supernatant (produced by dihydropyrimidine dehydrogenase) was determined by the second assay, which had a reaction mix (0.52 mL total volume) of 2 M KHCO₃, 0.5 M Tris-HCl buffer (pH 8.0), 0.1 M 2-ketoglutarate, and 12mM NADH (Xu, et al. 1994). The NADH appearance due to ammonium conversion of glutamate dehydrogenase was then followed spectrophotometrically over 60 minutes at 365 nm.

Pyridine nucleotide transhydrogenase

The specific activity of pyridine nucleotide transhydrogenase, a proton translocating enzyme for the reductive cofactor of dihydropyrimidine dehydrogenase, was measured to determine the prevalent cofactor of the reaction. The 1 mL reaction mixture contained 0.1 mM NADPH, 0.05 M Tris-HCl buffer (pH 7.5) and cell-free extract. (San Pietro, et al 1955) One milliliter of 0.1 mM of thionicotinamide adenine dinucleotide, or tNAD⁺, was added to initiate the enzyme activity for the assay. The conversion of tNAD⁺ to tNADH was followed at 400 nm and a molar absorptivity coefficient of $11.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used in determining the activity.

Isolation of Mutant

A protocol involving ethylmethane sulfonate (EMS) mutagenesis and 5-

fluoroorotic acid resistance was used (Watson and Holloway, 1976; Watrin et al., 1999). An exponential phase nutrient broth culture (5 mL) of each *P. syringae* was treated with 1% EMS for 90 minutes at 30°C without shaking. Each culture was diluted (1:30) in glucose minimal medium containing uracil (50 mg/L) and was shaken overnight at 30°C to allow outgrowth of potential mutants. The mutagenized cells were screened for possible penicillin resistant mutants by spreading mutagenized cells on glucose minimal medium plates containing 0.0001% (w/v) of penicillin and uracil.

Protein Assay

To obtain the concentration of protein in each sample for specific activity values, protein assays were performed on each sample of cell-free extract using the Bradford Method and lysozyme as the standard protein (Bradford, 1976). The Bradford dye was prepared by dissolving 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid in deionized water. Serial dilutions were performed with lysozyme as a standard. The Bradford dye was then added to these dilutions to allow the dye to bind to the protein. Absorbances were taken at 595 nm and used as a standard curve to interpolate concentrations of the cell-free extract samples. The cell-free extract samples were diluted to a 1:10 ratio with deionized water and Bradford dye was added to the samples for binding. Absorbances were taken at 595 nm and interpolated within the standard curves.

Results

It was the intent of this project to elucidate *P. syringae*'s (ATCC 12771) degradative pyrimidine biochemistry, and to observe the changes in the degradative pathway enzyme activities when the organism is grown on various nitrogen sources. This project was also intended to assess any changes in degradative enzyme activity through mutation of the organism in antibiotic resistant mutants. In order to determine if the organism reductively degrades pyrimidines, assays of the three reductive pathway enzymes were performed. Activity detected in these assays will implicate the expression of the enzymes of the reductive pathway.

Pseudomonas syringae ATCC 12771 was grown in modified Stanier liquid medium containing glucose as the carbon source and uracil, the starting substrate of the reductive pathway, as a nitrogen source. The cell-free extracts containing cellular protein were assayed for dihydropyrimidine dehydrogenase, dihydropyrimidinase and N-carbamoyl Beta-alanine amidohydrolase. The results of these assays showed activity for all three enzymes (Table 1). It was also shown, that for *P. syringae* grown on glucose (0.4% w/v) and uracil (0.2% w/v), the organism exhibits the highest activity in the dihydropyrimidinase reaction. Dihydropyrimidine dehydrogenase exhibits the lowest specific activity in this growth media, having 5.3% of the specific activity of the dihydropyrimidinase reaction (Table 1).

Table 1: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	3.54	[0.38]
Dihydropyrimidinase	66.10	[1.75]
N-carbamoyl- β -alanine amidohydrolase	9.01	[0.65]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles of product/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

In order to determine the effects of various nitrogen sources on the reductive enzyme activity of *P. syringae* ATC 12771, specific activities of the three enzymes were determined on eight other nitrogen sources using glucose as the carbon source. In order to determine control values for the specific activities of various nitrogen sources, reductive pathway enzyme assays were performed on the cell-free extracts of the organism grown on glucose (0.4% w/v) and no supplemented source of nitrogen (Table 2). The specific activities of the reductive enzymes of *P. syringae* grown on glucose and no supplemented source of nitrogen showed different trends from the those of the organism grown on glucose (0.4% w/v) and uracil (0.2% w/v). In this case, N-carbamoyl-Beta-alanine amidohydrolase exhibited the highest specific activity. By contrast, dihydropyrimidinase exhibited the lowest specific activity in this growth media, having roughly 0.017% of the specific activity of the N-carbamoyl-Beta-alanine amidohydrolase reaction (Table 2).

Reductive pathway specific enzyme activities were also determined for the organism grown on glucose (0.4% w/v) with thymine (0.2% w/v) as a nitrogen source (Table 3). These results were similar to the results of *P. syringae* grown on glucose and no supplemented source of nitrogen: the organism exhibits the highest activity in the N-carbamoyl-Beta-alanine amidohydrolase reaction. Dihydropyrimidinase exhibits the lowest specific activity in this growth media, having roughly 1.0 % of the specific activity of the N-carbamoyl-Beta-alanine amidohydrolase reaction (Table 3).

Table 2: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with no supplemental nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.42 [0.21]
Dihydropyrimidinase	0.0089 [0.0004]
N-carbamoyl- β -alanine amidohydrolase	52.10 [3.30]

The proteins were extracted from cells grown on 0.4% w/v carbon source and no supplemented source of nitrogen. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 3: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with thymine as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.08	[0.02]
Dihydropyrimidinase	0.011	[0.0017]
N-carbamoyl- β -alanine amidohydrolase	4.80	[0.80]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Reductive pathway specific enzyme activities were also determined for the organism grown on glucose (0.4% w/v) with either dihydrouracil (Table 4), dihydrothymine (Table 5), Beta-alanine (Table 6), Beta-amino-isobutyric acid (Table 7), ammonium sulfate (Table 8), or cytosine (Table 9) as nitrogen sources (0.2% w/v). For *P. syringae* grown on these nutrient sources, highest specific activity seems to be consistently in the dihydropyrimidase reaction and lowest in the dihydropyrimidine dehydrogenase reaction. The dihydropyrimidine dehydrogenase reaction is consistently several orders of magnitude lower in specific activity than dihydropyrimidinase (Tables 4-9).

The overall specific activity of the reductive enzymes of *P. syringae* was highest for the organism grown on glucose and dihydrouracil. Conversely, the lowest overall specific activity of the reductive enzymes was found for *P. syringae* grown on glucose and ammonium sulfate (Tables 4 and 8).

In order to determine the effects that changes in the carbon nutrient source have on the specific activity of *P. syringae*'s (ATCC 12271) reductive enzymes, the above assays were replicated using succinate as a carbon source in place of glucose. The control for these assays was determined using succinate (0.4% w/v) and no supplemental source of nitrogen (Table 10). The trend of the resulting specific activities for the reductive enzymes of *P. syringae* grown on this nutrient source remained the same with N-carbamoyl-Beta-alanine amidohydrolase exhibiting the highest specific activity and dihydropyrimidinase exhibiting the lowest specific activity. The overall specific activities for this nutrient source, however, were

Table 4: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrouracil as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	1.42 [0.30]
Dihydropyrimidinase	140.50 [11.90]
N-carbamoyl- β -alanine amidohydrolase	71.32 [1.18]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 5: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrothymine as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.74 [0.33]
Dihydropyrimidinase	142.90 [0.45]
N-carbamoyl- β -alanine amidohydrolase	29.17 [3.69]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 6: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-alanine as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.50	[0.05]
Dihydropyrimidinase	128.80	[9.20]
N-carbamoyl- β -alanine amidohydrolase	27.81	[3.20]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 7: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-aminoisobutyric acid as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.19	[0.03]
Dihydropyrimidinase	87.30	[26.10]
N-carbamoyl- β -alanine amidohydrolase	7.99	[0.39]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 8: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with ammonium sulfate as nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.05	[0.009]
Dihydropyrimidinase	23.19	[2.10]
N-carbamoyl- β -alanine amidohydrolase	28.32	[11.98]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 9: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with cytosine as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.10	[0.02]
Dihydropyrimidinase	82.83	[7.10]
N-carbamoyl- β -alanine amidohydrolase	41.06	[8.80]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 10: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with no supplemental nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.15 [0.09]
Dihydropyrimidinase	0.0009 [0.0001]
N-carbamoyl- β -alanine amidohydrolase	1.20 [0.30]

The proteins were extracted from cells grown on 0.4% w/v carbon source and no supplemented nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

significantly lower than those from glucose as a carbon source (Table 2 and 10).

Dihydropyrimidine dehydrogenase exhibited one third of the specific activity exhibited with the organism grown on glucose; dihydropyrimidinase exhibited one-tenth of the activity, and N-carbamoyl-Beta-alanine amidohydrolase exhibited one-fiftieth of the activity (Tables 2 and 10).

Reductive pathway enzyme specific activities were also determined for the organism grown on succinate (0.4% w/v) with thymine (0.2% w/v) as a nitrogen source (Table 11). These results were similar to the results of *P. syringae* grown on succinate (0.4% w/v) and no supplemented source of nitrogen. The organism exhibits the highest activity in the N-carbamoyl-Beta-alanine amidohydrolase reaction and dihydropyrimidinase exhibits the lowest specific activity in this growth media, having roughly 1.0 % of the specific activity of the N-carbamoyl-Beta-alanine amidohydrolase reaction (Table 11).

The specific activities were also determined using succinate (0.4% w/v) as a carbon source with either uracil (Table 12), dihydrouracil (Table 13), dihydrothymine (Table 14), Beta-alanine (Table 15), Beta-amino-isobutyric acid (Table 16), ammonium sulfate (Table 17), or cytosine (Table 18) as nitrogen sources. The trends in specific activities for the enzymes of *P. syringae* grown on these nutrient sources and those grown on the same nitrogen sources and glucose. For the reductive enzymes of *P. syringae* grown on succinate nutrient sources, the highest specific activity was consistently found in the dihydropyrimidinase reaction and lowest in the dihydropyrimidine dehydrogenase reaction. The dihydropyrimidine dehydrogenase

Table 11: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with thymine as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.66 [0.26]
Dihydropyrimidinase	0.03 [0.008]
N-carbamoyl- β -alanine amidohydrolase	4.10 [0.02]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 12: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with uracil as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.33 [0.002]
Dihydropyrimidinase	166.50 [17.70]
N-carbamoyl- β -alanine amidohydrolase	1.04 [0.23]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 13: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with dihydrouracil as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.31	[0.02]
Dihydropyrimidinase	95.30	[2.30]
N-carbamoyl- β -alanine amidohydrolase	7.45	[1.55]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 14: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with dihydrothymine as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.66 [0.02]
Dihydropyrimidinase	327.60 [29.20]
N-carbamoyl- β -alanine amidohydrolase	11.09 [3.79]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 15: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with Beta-alanine as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.24 [0.01]
Dihydropyrimidinase	195.80 [39.90]
N-carbamoyl- β -alanine amidohydrolase	9.08 [0.40]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 16: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with Beta-aminoisobutyric acid as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.23 [0.04]
Dihydropyrimidinase	112.50 [15.50]
N-carbamoyl- β -alanine amidohydrolase	2.23 [0.75]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 17: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with ammonium sulfate as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.32 [0.05]
Dihydropyrimidinase	165.10 [20.10]
N-carbamoyl- β -alanine amidohydrolase	7.71 [0.41]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 18: Pyrimidine reductive pathway enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with cytosine as a nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.58 [0.01]
Dihydropyrimidinase	222.70 [30.70]
N-carbamoyl- β -alanine amidohydrolase	15.61 [2.96]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

reaction is consistently several orders of magnitude lower in specific activity than dihydropyrimidinase (Tables 12-18).

The overall specific activity of the reductive enzymes of *P. syringae* was highest for the organism grown on succinate and dihydrouracil (Table 13). Conversely, the lowest overall specific activity of the reductive enzymes were those of *P. syringae* grown on succinate and ammonium sulfate (Tables 13 and 17).

There are no trend differences for higher and lower specific activities between glucose and succinate as nutrient sources with the exception of the aforementioned lowered activities for succinate and no supplemental nutrient source (Tables 2 and 10).

This project also investigated the optimal substrate and cofactor for the dihydropyrimidine dehydrogenase reaction in *Pseudomonas syringae*. Uracil and thymine both serve as substrates for this reaction, and both NADH and NADPH are said to contribute to the reduction of the double bond between the second and third carbons of uracil or thymine (Figures 3 and 4). Assays were run to determine the difference between the specific activities using uracil and NADH as substrate and cofactor, thymine and NADH as substrate and cofactor, uracil and NADPH as substrate and cofactor, and thymine and NADPH as substrate and cofactor.

This procedure was done for the dihydropyrimidine dehydrogenase enzyme of *P. syringae* grown on glucose (0.4% w/v) as a carbon source and no supplemented source of nitrogen. This serves as a control and has a minimal probability of substrate competition due to nitrogen nutrient sources.

The optimal substrate and cofactor was determined to be uracil and NADPH, respectively. The specific activity of dihydropyrimidine dehydrogenase utilizing NADPH as the cofactor was over two times higher than the specific activity of the enzyme utilizing NADH as the cofactor. The specific activity of dihydropyrimidine dehydrogenase was increased marginally (one-tenth) when uracil was used as a substrate than with thymine used as a substrate (Table 19).

The optimal substrate and cofactor for the dihydropyrimidine dehydrogenase reaction in *P. syringae* was also found for the organism grown on glucose as a carbon source (0.4% w/v) and either uracil (Table 20), thymine (Table 21), dihydrouracil (Table 22), dihydrothymine (Table 23), Beta-alanine (Table 24), Beta-aminoisobutyric acid (Table 25), ammonium sulfate (Table 26) or cytosine (Table 27) as a nitrogen source (0.2% w/v for all nitrogen sources).

The optimal substrate and cofactor was determined to be uracil and NADPH, respectively. The trend of specific activity in dihydropyrimidine dehydrogenase was higher when NADPH was the cofactor than when NADH was the cofactor. There was a trend of higher specific activity of dihydropyrimidine dehydrogenase when uracil was used as a substrate than with thymine used as a substrate (Tables 20-27).

Table 19: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with no supplemental source of nitrogen and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.42	[0.21]
NADH / Thymine	0.39	[0.02]
NADPH / Uracil	1.03	[0.06]
NADPH / Thymine	0.90	[0.08]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 20: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	3.54	[0.38]
NADH / Thymine	0.70	[0.12]
NADPH / Uracil	0.48	[0.05]
NADPH / Thymine	0.49	[0.10]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 21: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with thymine as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.08	[0.02]
NADH / Thymine	0.17	[0.06]
NADPH / Uracil	0.50	[0.06]
NADPH / Thymine	0.45	[0.06]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 22: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrouracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	1.42	[0.30]
NADH / Thymine	0.76	[0.11]
NADPH / Uracil	4.70	[0.15]
NADPH / Thymine	4.54	[0.16]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 23: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with dihydrothymine as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.74	[0.33]
NADH / Thymine	0.11	[0.06]
NADPH / Uracil	1.43	[0.01]
NADPH / Thymine	1.47	[0.004]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 24: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-alanine as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.49	[0.04]
NADH / Thymine	0.58	[0.06]
NADPH / Uracil	0.71	[0.02]
NADPH / Thymine	0.61	[0.11]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 25: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with Beta-aminoisobutyric acid as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.19	[0.03]
NADH / Thymine	0.11	[0.03]
NADPH / Uracil	0.30	[0.07]
NADPH / Thymine	0.12	[0.02]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 26: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with ammonium sulfate as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.10	[0.02]
NADH / Thymine	0.15	[0.04]
NADPH / Uracil	0.17	[0.04]
NADPH / Thymine	0.16	[0.002]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 27: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with cytosine as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity
NADH / Uracil	0.10 [0.02]
NADH / Thymine	0.15 [0.04]
NADPH / Uracil	0.17 [0.04]
NADPH / Thymine	0.16 [0.002]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

The optimal substrate and cofactor was also determined for the dihydropyrimidine dehydrogenase reaction in *P. syringae* grown on succinate (0.4% w/v) as a carbon source and no supplemented source of nitrogen. This serves as a control and has a minimal probability of substrate competition due to nitrogen nutrient sources.

The optimal substrate and cofactor was determined to be uracil and NADPH, respectively. The specific activity of dihydropyrimidine dehydrogenase utilizing NADPH as the cofactor was over three times higher than the specific activity of the enzyme utilizing NADH as the cofactor. The specific activity of dihydropyrimidine dehydrogenase was not significantly different when uracil was used as a substrate than with thymine used as a substrate (Table 28).

The optimal substrate and cofactor for the dihydropyrimidine dehydrogenase reaction in *P. syringae* was also found for the organism grown on succinate as a carbon source (0.4% w/v) and either uracil (Table 29), thymine (Table 30), dihydrouracil (Table 31), or ammonium sulfate (Table 32) as a nitrogen source (0.2% w/v for all nitrogen sources).

The optimal substrate and cofactor was determined to be uracil and NADPH, respectively. The trend of specific activity of dihydropyrimidine dehydrogenase utilizing NADPH as the cofactor was larger than the specific activity of the enzyme utilizing NADH as the cofactor. The trend of specific activity of dihydropyrimidine dehydrogenase was higher when uracil was used as a substrate than with thymine used as a substrate (Tables 29-32).

Table 28: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with no supplemental source of nitrogen and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity
NADH / Uracil	0.15 [0.09]
NADH / Thymine	0.23 [0.04]
NADPH / Uracil	0.52 [0.12]
NADPH / Thymine	0.44 [0.22]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 29: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.33	[0.002]
NADH / Thymine	0.33	[0.06]
NADPH / Uracil	1.11	[0.03]
NADPH / Thymine	0.47	[0.02]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 30: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with thymine as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.66	[0.15]
NADH / Thymine	0.60	[0.26]
NADPH / Uracil	1.21	[0.28]
NADPH / Thymine	0.80	[0.08]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 31: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with dihydrouracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.31	[0.02]
NADH / Thymine	0.45	[0.01]
NADPH / Uracil	0.85	[0.01]
NADPH / Thymine	0.50	[0.07]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Table 32: Dihydropyrimidine dehydrogenase enzyme activities for *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing succinate as the carbon source with ammonium sulfate as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor / Substrate	Specific Activity	
NADH / Uracil	0.31	[0.04]
NADH / Thymine	0.24	[0.01]
NADPH / Uracil	0.80	[0.11]
NADPH / Thymine	0.77	[0.12]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Though the optimal reducing cofactor of the organism was determined to be NADPH, it is not known if NADPH exists and is available in the cell. The existence of NADPH in the cell was determined by performing a pyridine nucleotide transhydrogenase enzyme assay on *P. syringae* cells grown on glucose (0.4% w/v) and uracil (0.2% w/v). Pyridine nucleotide transhydrogenase catalyzes the transfer of a hydride ion from NADPH to NAD^+ to yield NADH. Specific enzyme activity detected for pyridine nucleotide transhydrogenase verifies the presence of NADPH available in the cell (French, et al 1997).

Enzyme activity was detected in an enzyme assay with pyridine nucleotide transhydrogenase and no substrate supplemented in the assay mixture. It was verified that NADPH actually exists in the cell (Table 33).

Finally, this project was also intended to determine the effects of penicillin resistance on the expression of the reductive enzymes of *Pseudomonas syringae* ATCC 12271. Reductive pathway specific enzyme activities were determined for the organism grown on glucose (0.4% w/v) with uracil (0.2% w/v) as a nitrogen source and penicillin (0.0001% w/v) (Table 34). These results were similar to the results of *P. syringae* grown on glucose and uracil (Table 1). The organism exhibits the highest activity in the dihydropyrimidinase reaction and dihydropyrimidine dehydrogenase exhibits the lowest specific activity in this growth media, having one-fourteenth of the specific activity of the dihydropyrimidinase reaction (Tables 1 and 34).

Table 33: Pyridine nucleotide transhydrogenase specific activity for *Pseudomonas syringae* ATCC 12771 grown in minimal medium.

Carbon Source	Nitrogen Source	Specific Activity
Glucose	Uracil	9.30 [1.32]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Overall, the mutant organism expressed lower dihydropyrimidine dehydrogenase and N-carbamoyl-Beta-alanine amidohydrolase activities than the parent strain (Tables 1 and 34). The mutant expressed roughly five and a half times higher activity than the non-mutated parent organism in the dihydropyrimidinase reaction. The optimal substrate and cofactor for the dihydropyrimidine dehydrogenase reaction in penicillin resistant *P. syringae* for the organism grown on glucose as a carbon source (0.4% w/v) and uracil as a nitrogen source (0.2% w/v) was also determined to be uracil and NADPH, respectively. The trend of specific activity of dihydropyrimidine dehydrogenase utilizing NADPH as the cofactor was higher than the specific activity of the enzyme utilizing NADH as the cofactor. There was no significant change of specific activity of dihydropyrimidine dehydrogenase when uracil was used as a substrate than with thymine used as a substrate (Table 35).

Table 34: Pyrimidine reductive pathway enzyme activities for penicillin resistant *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source.

Enzyme	Specific Activity	
Dihydropyrimidine dehydrogenase	0.27	[0.08]
Dihydropyrimidinase	372.20	[19.90]
N-carbamoyl- β -alanine amidohydrolase	4.94	[0.58]

The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from the three aforementioned samples. The standard deviation is indicated in [].

Table 35: Dihydropyrimidine Dehydrogenase enzyme activities for penicillin resistant *Pseudomonas syringae* ATCC 12771 grown in minimal medium containing glucose as the carbon source with uracil as a nitrogen source and either NADH or NADPH as a reducing cofactor.

Cofactor and Substrate	Specific Activity	
NADH uracil	0.27	[0.08]
NADH thymine	0.86	[0.19]
NADPH uracil	0.55	[0.04]
NADPH thymine	0.64	[0.07]

The assay solution consisted of either uracil or thymine as a substrate and either NADH or NADPH as a starting material. The proteins were extracted from cells grown on 0.4% w/v carbon source and 0.2% w/v nitrogen source. Specific activities are measured as nanomoles/minute/milligram of protein at 30 degrees Celsius. Each value is the mean of the independent values obtained from three samples. The standard deviation is indicated in [].

Discussion

The purpose of project was to determine if *P. syringae* ATCC 12271 expresses the enzymes of the reductive pathway for degradation of pyrimidines. Based on the results in the aforementioned assay determinations, it was concluded that *P. syringae* does in fact express reductive pathway enzymes. It can also be determined that the enzymes' activities can be regulated.

The highest specific enzyme activity was consistently shown to be in the dihydropyrimidine dehydrogenase reaction in *P. syringae* cells grown on the nitrogen source uracil. Dihydropyrimidine dehydrogenase exhibits the lowest specific activity in cells of *P. syringae* grown on ammonium sulfate. These results are expected, given that uracil is the substrate for the dihydropyrimidine dehydrogenase reaction. It is speculated that this is due to the higher concentration of uracil available in the cell from uptake of the nutrient sources.

In the dihydropyrimidinase reaction, the highest specific activity is observed when the organism is grown on succinate and dihydrouracil. This is expected, given that dihydrouracil is the substrate for the dihydropyrimidinase reaction. It is speculated that this is due to a higher concentration of dihydrouracil available in the cell from the uptake of nutrient sources. In this reaction, the higher activity was also observed when the organism was grown on succinate. This is possibly due to a need to break down pyrimidines for carbon sources. Succinate has not been shown to be actively transported across the cell membrane, which may imply a shortage of carbon sources and an increase in substrate concentration (with dihydrouracil). The lowest specific activity for this reaction was observed when the organism was grown without

a supplemental source of nitrogen (and grown on both glucose and succinate). It is speculated that this is due to a lack of substrate for the reaction.

The highest enzyme specific activity in the N-carbamoyl-Beta-alanine amidohydrolase reaction was observed for the organism grown on glucose and dihydrouracil. The lowest activity was observed for the organism grown without a supplemented nitrogen source. It is speculated that the high activity of NCBA-A in the organism grown on glucose and dihydrouracil is due to the rapid conversion of dihydrouracil to N-carbamoyl-Beta-alanine by dihydropyrimidinase in the cell. This would provide the substrate for the reaction and result in high activity.

Another goal of this investigation was to determine the optimal substrate and reducing cofactor for the dihydropyrimidine dehydrogenase reaction. The optimal substrate of the reaction was determined to be uracil. This is expected, given that uracil is the substrate of the reaction and that higher activity is observed for this reaction when the organism is grown on uracil as opposed to thymine (O'Donovan, et al 1970). The optimal reducing cofactor of the reaction was determined to be NADPH. This can also be expected, given that NADPH is a stronger reducing cofactor due to the highly reactive phosphate group that transfers the hydride and reduces the 3rd and 4th carbon in uracil (Campbell, 1957, 1958). However, it is interesting to note that normally eukaryotic organisms, not prokaryotic such as *P. syringae*, use NADPH as a cofactor in the reaction.

In order for the organism to have NADPH in the cell, it must use the pentose phosphate pathway. It is not known whether the organism actually uses the pentose phosphate pathway, which gives rise to the uncertainty that the higher specific

activity due to NADPH used as a cofactor actually occurs in nature. The existence of NADPH, therefore, was verified in a pyridine nucleotide transhydrogenase enzyme assay performed on *P. syringae* grown on glucose (0.4% w/v) and uracil (0.2% w/v). Specific enzyme activity was observed for this reaction. It was determined, therefore, that the cell of the organism does contain NADPH, and that it is readily available in the cell.

The effect of mutagenesis on the reductive enzyme activity of *P. syringae* ATCC 12271 was also determined. The organism, grown on glucose and uracil, exhibited the same trend in activity as the parent strain grown on glucose and uracil. Overall, the mutant organism expressed roughly 5.6 times higher specific activity than the parent strain in the dihydropyrimidine dehydrogenase reaction and the N-carbamoyl-Beta-alanine amidohydrolase reactions. It expressed roughly one-fifteenth of the activity of the parent strain in the dihydropyrimidinase reaction. It was concluded, from these results, that there was an effect on the reductive enzyme activity of *P. syringae* ATCC 12271 with mutagenesis. It is speculated that the mutation occurred on the level of transcription, and possibly could have occurred at the level of DNA synthesis.

It was also concluded that the optimal substrate and cofactor for the dihydropyrimidine dehydrogenase reaction in the mutant was uracil and NADPH, respectively. It was determined, then, that there was no change in expression of the reduction of uracil in the dihydropyrimidine dehydrogenase reaction with mutagenesis of penicillin resistance.

References

- Bradford, M.M. (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* **72**, 248-254.
- Campbell, L.L., Jr. (1957) Reductive degradation of pyrimidines. III. Purification and properties of dihydrouracil dehydrogenase. *J. Biol. Chem.* **227**, 693-700.
- Campbell, L.L., Jr. (1958) Reductive degradation of pyrimidines. IV. Purification and properties of dihydrouracil hydrase. *J. Biol. Chem.* **233**, 1236-1240.
- Chu, C-P, and West, T.P. (1990) Pyrimidine biosynthetic pathway of *Pseudomonas fluorescens*. *J. Gen. Microbiol.* **136**, 875-880.
- French, C.E., Boonstra, B., Bufton K.A.j., and Bruce N.C. (1997) Cloning, sequence, and properties of the soluble pyridine nucleotide transhydrogenase of *Pseudomonas fluorescens*. *J. Bacteriol.* **79**, 2765-2765
- Naidu R.; Baskaran S.; Kookana R.S.¹ Pesticide fate and behaviour in Australian soils in relation to contamination and management of soil and water: a review. *Australian Journal of Soil Research*, 20 August 1998, vol. 36, no. 5, pp. **715-764**(50) CSIRO Publishing
- O'Donovan, G.A., and Neuhard, J. (1970) Pyrimidine metabolism in microorganisms. *Bacteriol. Rev.* **34**, 278-343.
- Prescott, L.M., and Jones, M.E. (1969) Modified methods for the determination of carbamyl aspartate. *Anal. Biochem.* **32**, 408-419.
- Stanier, R.Y. (1947) Simultaneous adaptation: a new technique for the study of metabolic pathways. *J. Bacteriol.* **54**, 339-348.
- San Pietro, A., Kaplan, N.O., and Colowick, S.P. (1955) Pyridine nucleotide transhydrogenase. VI. Mechanism and stereospecificity of the reaction in *Pseudomonas fluorescens*. *J. Biol. Chem.* **212**, 941-952.
- Tamaki, N. and Mizutani, N. (1987) Purification and properties of β -ureidopropionase from the rat liver. *Eur. J. Biochem.* **169**, 21-26.
- Vogels, G. D., and van der Drift, C. (1976) Degradation of purines and pyrimidines by microorganisms. *Bacteriol. Rev.* **40**, 403-468.

Watson, J.M. and Holloway, B.W. (1976) Suppressor mutations in *Pseudomonas aeruginosa*. J. Bacteriol. **125**, 780-786.

Wang, T.P., and Lampen, J.O. (1951) Metabolism of pyrimidines by a soil bacterium. J. Biol. Chem. **194**, 775-783.

West, T.P., Shanley, M.S., and O'Donovan, G.A. (1982) Improved colorimetric procedure for quantitating N-carbamoyl- β -alanine with minimum dihydrouracil interference. Anal. Biochem. **122**, 345-347.

West, T.P., Traut, T.W., Shanley, M.S., and O'Donovan, G.A. (1985) A *Salmonella typhimurium* strain defective in uracil catabolism and β -alanine synthesis. J. Gen. Microbiol. **131**, 1083-1090.

Xu, G., and West, T.P. (1992) Reductive catabolism of pyrimidine bases by *Pseudomonas stutzeri*. J. Gen. Microbiol. **138**, 2459-2463.

Xu, G., and West, T.P. (1994) Characterization of dihydropyrimidinase from *Pseudomonas stutzeri*. Arch Microbiol. **161**, 70-74.

Acknowledgements

The University of Tennessee at Chattanooga Provost Student Research Fund

The University of Tennessee at Chattanooga Grote Fund

The University of Tennessee at Chattanooga Chemistry Department

The University of Tennessee at Chattanooga Summer Research Program

Manuel F. Santiago, Ph.D., Project Director

Gail M. Meyer, Ph.D., Professor of Chemistry

Jim Narramore, Professor of Chemistry

H. Douglas Kutz, Ph.D., Department Examiner

David Sachsman, Ph.D. , Department Examiner