

**REDUCTIVE CATABOLISM OF PYRIMIDINES BY *PSEUDOMONAS
LEMONNIERI***

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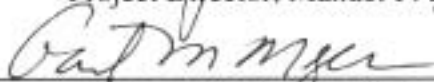
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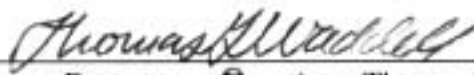
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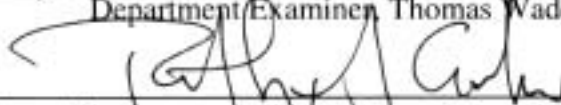
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ABSTRACT

Metabolic reactions are vital to the survival of cells. There are two major categories of these reactions, catabolic and biosynthetic. Catabolic reactions break down molecules to utilize the constituent parts, and biosynthetic reactions build larger biomolecules. The pyrimidine reductive catabolic pathway is one of two mechanisms that can be utilized by microorganisms to degrade nucleic acid bases for their nitrogen. The other pyrimidine catabolic pathway is oxidative and its end products include urea and malonic acid. It has been previously shown that some *Pseudomonas* species are able to utilize pyrimidines as sole nitrogen sources, and this could be a mechanism for either negative or positive growth control. *Pseudomonas* species have been found to be opportunistic pathogens and are agriculturally and environmentally important. Therefore, the reductive pathway was studied in *Pseudomonas lemonnieri* ATCC 12983 and an isolated antibiotic-resistant mutant strain BS0100. The reductive pathway enzymes (dihydropyrimidine dehydrogenase, dihydropyrimidinase, and *N*-carbamoyl- β -alanine amidohydrolase) were assayed to determine their specific activities after the microorganism had been grown in Stanier minimal media with either glucose or succinate serving as the carbon source, and the nitrogen source was varied. The three reductive pathway enzymes were detected in both the parent and mutant strains. Specific activities in the two strains showed marked differences based on the different carbon and nitrogen sources. There is evidence that the enzyme dihydropyrimidine dehydrogenase has specificity for the nicotinamide cofactor NADPH. Also, the organism was found to be able to degrade cytosine by utilizing the enzyme cytosine deaminase. From this study, it was determined that *P. lemonnieri* ATCC 12983 and the mutant strain BS0100 utilized the reductive pathway to degrade pyrimidines.

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INTRODUCTION

The two types of metabolic reactions, biosynthetic and catabolic, are usually coupled together so that the energy released from the catabolism of biomolecules to their smaller components can be utilized for the biosynthesis of larger biomolecules that are essential to the cell. Some essential nutrients to the cell include proteins and nucleic acids, both of which contain nitrogen. Nitrogen is an important element in biological compounds since it is one of the building blocks of amino acids used to make proteins and nucleic acid bases used in the production of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Therefore, when amino acids and nucleic acid bases are catabolized, they release nitrogen, which can be reutilized to build other important biomolecules (O'Donovan and Neuhard, 1970). Much work has been carried out concerning amino acid metabolism, but there have not been many studies thus far on the topic of pyrimidine base metabolism. Pyrimidine base biosynthesis, however, has been more widely studied than pyrimidine catabolism (O'Donovan and Neuhard, 1970; Vogels and van der Drift, 1976).

When a living cell is starved of nitrogen, it will begin to express genes that allow the cell to utilize nitrogen that may be tied up in other biomolecules. Pyrimidines are one such biomolecule that could be catabolized to liberate nitrogen from within the aromatic ring. Within the living cell, pyrimidines can be degraded by two different catabolic pathways (Vogels and van der Drift, 1976). Some organisms degrade pyrimidines oxidatively, where uracil is catabolized in a two-step pathway (Wang and Lampen 1951*a*; Wang and Lampen 1951*b*; Hayaishi and Kornberg, 1952; Hayaishi and Kornberg, 1951). The first step, which oxidizes uracil to barbituric acid, is catalyzed by the enzyme uracil

dehydrogenase (EC 1.2.99.1). The second enzymatic step catabolizes barbituric acid to urea and malonic acid, and this reaction is catalyzed by barbiturase (EC 3.5.2.1). The oxidative catabolism of pyrimidines has been found to exist in *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Chlamydia*, and *Enterobacter* (Hayaishi and Kornberg, 1951; Hayaishi and Kornberg, 1952; McClarty and Qin, 1993; Vogels and van der Drift, 1976; Wang and Lampen 1951*a*; Wang and Lampen 1951*b*).

The second pathway that is known to exist in living cells to degrade pyrimidines is the reductive pathway. This pathway is more prevalent in bacteria, and it catabolizes pyrimidines in a three-step enzymatic mechanism. The enzymes of the pathway can degrade either uracil (Figure 1) or thymine (Figure 2). Cytosine can be converted to uracil by way of the enzyme cytosine deaminase in order to enter the reductive pathway. The first step of the pathway is catalyzed by dihydropyrimidine dehydrogenase (EC 1.3.1.2), which reduces uracil to dihydrouracil. This enzyme requires a nicotinamide cofactor, either reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH). Generally, eukaryotes utilize NADPH as the enzyme cofactor, whereas NADH is usually preferred by prokaryotes (Campbell, 1957; Campbell, 1958). The enzymatic function of dihydropyrimidine dehydrogenase is particularly interesting because cancer patients who are deficient in this essential enzyme can develop a toxic reaction to the 5-flourouracil used in chemotherapy treatments (Van Kuilenburg et. al., 1996). The dihydrouracil ring is then broken to form *N*-carbamoyl- β -alanine. This step is catalyzed by the enzyme dihydropyrimidinase (EC 3.5.2.2). This enzyme is of particular commercial interest because it has the capability of producing synthetic D-amino acids. The final step in the reductive pathway breaks down

N-carbamoyl- β -alanine into β -alanine, carbon dioxide, and ammonia. This reaction is catalyzed by the enzyme *N*-carbamoyl- β -alanine amidohydrolase (EC 3.5.1.6) (Vogels and van der Drift, 1976).

The reductive pathway has been detected in some eukaryotes, mammals included (Fritzson and Pihl, 1957; Fritzson, 1957; Sanno et. al., 1970; Traut and Loechel; 1984). The second enzyme of the reductive pathway, dihydropyrimidinase, has been found to be active in both the liver and the peripheral blood lymphocytes of humans (Naguib et. al., 1985), and it has been purified and characterized from the liver tissue of other mammals (Lu et. al., 1992). This activity in conjunction with investigations of this enzyme in the human fetal brain suggest that pyrimidine catabolism may be active in humans (Hamajima et. al., 1996). In addition to humans, the reductive catabolism of pyrimidines has been detected in *Euglena gracilis* and *Chlorella fusca* (Wasternack et. al., 1977; Tsai and Axelrod, 1965; Knutsen, 1972).

Prokaryotes have also been investigated for the activity of the reductive pathway (O'Donovan and Neuhard, 1970; Vogels and van der Drift, 1976). The microorganism *Escherichia coli* was studied and found to have the capability of actively degrading pyrimidines through the reductive pathway (West, 1998). In addition to *E. coli*, *Salmonella typhimurium* was investigated to determine if the uracil reductive pathway was present (West et. al., 1985). In this study it was found that the microorganism grew poorly on uracil, but the reductive pathway enzymes were active. Also, in the same study it was reported that a mutant of the second enzyme dihydropyrimidinase was isolated. This confirmed that the reductive pathway exists in *S. typhimurium* (West et. al., 1985).

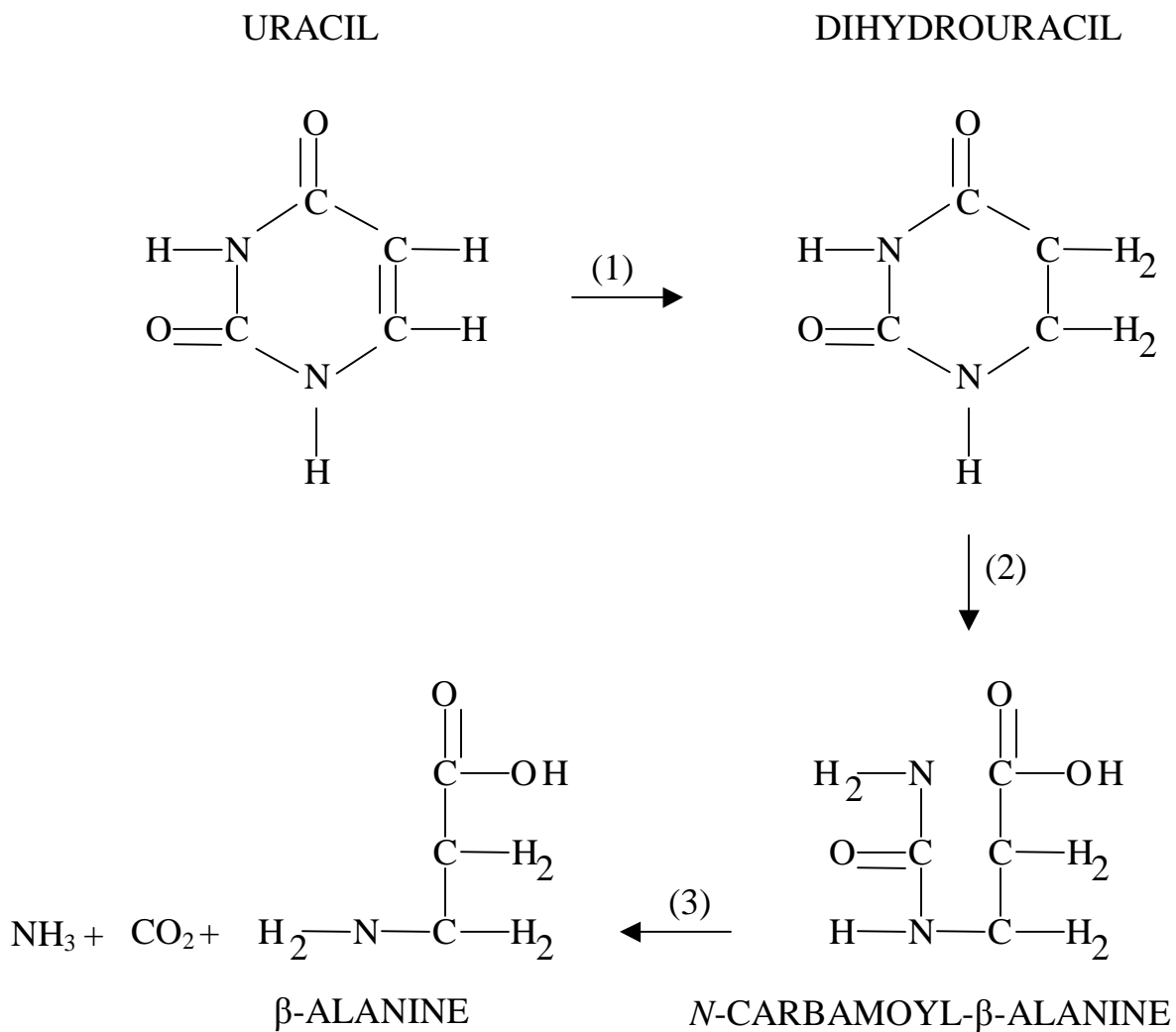


FIGURE 1: THE URACIL REDUCTIVE PATHWAY

(1) The reduction of uracil to dihydrouracil is catalyzed by dihydropyrimidine dehydrogenase.

(2) The dihydrouracil ring is broken to form *N*-carbamoyl- β -alanine by dihydropyrimidinase.

(3) The conversion of *N*-carbamoyl- β -alanine to β -alanine, carbon dioxide, and ammonia is catalyzed by *N*-carbamoyl- β -alanine amidohydrolase.

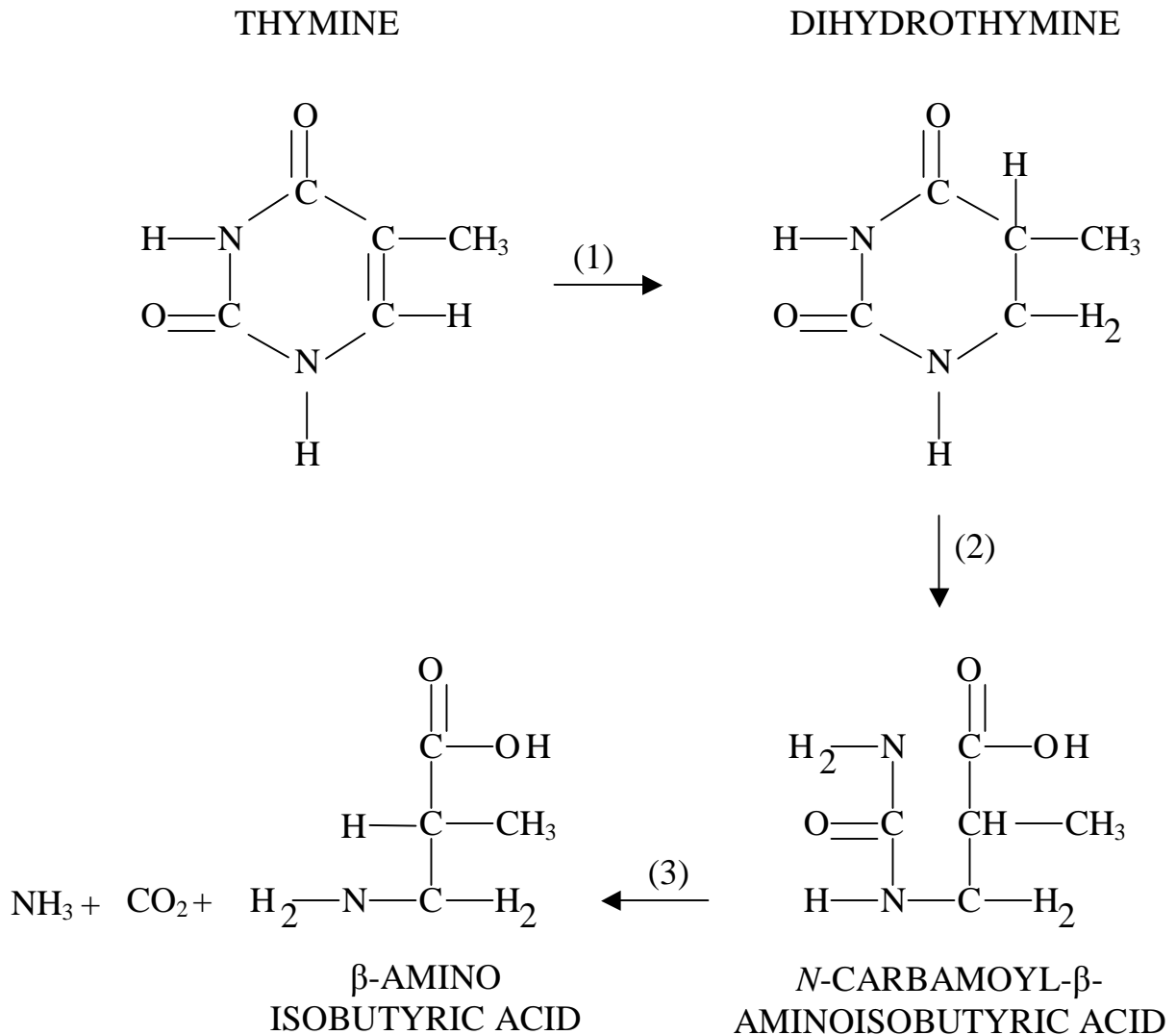


FIGURE 2: THE THYMINE REDUCTIVE PATHWAY

(1) The reduction of thymine to dihydrothymine is catalyzed by dihydropyrimidine dehydrogenase. (2) The dihydrothymine ring is broken to form *N*-carbamoyl- β -aminoisobutyric acid by dihydropyrimidinase. (3) The conversion of *N*-carbamoyl- β -aminoisobutyric acid to β -aminoisobutyric acid, carbon dioxide, and ammonia is catalyzed by *N*-carbamoyl- β -alanine amidohydrolase.

In addition to the reductive catabolism of pyrimidines, a similar pathway exists in the microorganism *Blasterobacter* that breaks down dihydropyrimidines and cyclic imides (Ogawa et. al., 1996). The enzyme dihydropyrimidinase hydrolyzes the bases and imidase breaks open the hydantoin ring. In addition to the presence of these two enzymes, amidohydrolase was found to be thermostable in *Blasterobacter* (Ogawa et. al., 1994). The enzyme dihydropyrimidinase in *Agrobacterium tumefaciens* catalyzes the production of *N*-carbamoyl-D-amino-acids rather than *N*-carbamoyl- β -amino-acids (Durham and Weber, 1995).

Species of the microorganism *Pseudomonas* are opportunistic pathogens and have been isolated from plants, animals, and humans. They have been found in soil, water, and sediment (Givskov et. al., 1994). For this reason, the regulation of their growth could be important both medically and environmentally. Species of *Pseudomonas* have been found to be able to utilize oil as a carbon source (Tortora, G., Funke, B., and Case, C., 2001), which could potentially be helpful in the cleanup of an oil spill. However, the microorganism grows too slowly under normal conditions to be effective. Therefore, a test beach was created and treated with nitrogen fertilizers. This enabled the bacteria to grow at a fast enough rate to successfully degrade the oil and leave the beach clean (Tortora, G., Funke, B., and Case, C., 2001).

Earlier investigations have shown that the reductive catabolism of pyrimidines is the prevalent pathway in selected species of *Pseudomonas*. These include *Pseudomonas pickettii*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, and *Pseudomonas fluorescens* (West, 1994; Ogawa et. al., 1994; West, 1991; Colowick et. al., 1955; Kim

and West, 1991; Xu and West, 1992; Santiago and West, 1999). When *P. pseudoalcaligenes* cells were grown using pyrimidines as nitrogen sources, the reductive pathway enzyme activities were increased (West, 1994). Growth on uracil, thymine, and dihydrothymine increased dihydropyrimidine dehydrogenase activity by at least three-fold, and growth on dihydrouracil caused the enzyme activity of dihydropyrimidinase to increase 26-fold (West, 1994).

An investigation of the reductive pathway in cells of *P. putida* revealed that the enzyme with highest activity was for dihydropyrimidinase, and dihydropyrimidine dehydrogenase had the lowest activity (Ogawa et. al., 1994). When analyzing the activity of the enzyme dihydropyrimidine dehydrogenase, it was noted that the activity was present only when the nicotinamide cofactor NADPH was used rather than NADH (Ogawa et. al., 1994). The reductive pathway in *P. chlororaphis* was studied when the nitrogen source was varied (West, 1991). It was determined from this investigation that dihydropyrimidine dehydrogenase and dihydropyrimidinase had higher enzyme activities when uracil, thymine, dihydrouracil, or dihydrothymine was used as the nitrogen source. In addition, it was found that *P. chlororaphis* has a nicotinamide cofactor specificity for NADH rather than NADPH, but NADPH activity was detectable (West, 1991). This could be due to the presence of pyridine nucleotide transhydrogenase (EC 1.6.1.1), which reduces NAD⁺ by means of NADPH (Colowick et. al., 1955).

One of the known *Pseudomonas* opportunistic pathogens, *P. aeruginosa*, has also been investigated for the reductive pathway. *Pseudomonas aeruginosa* was found to be able to utilize uracil, thymine, and their intermediates as nitrogen sources, and all three reductive pathway enzymes showed activity. When uracil was provided as the sole

nitrogen source, the three enzyme activities were significantly increased. Utilizing uracil, β -alanine, and β -amino-isobutyric acid as nitrogen sources resulted in the highest growth rate of the microorganism. The activities of dihydropyrimidinase and *N*-carbamoyl- β -alanine amidohydrolase were increased when thymine served as the nitrogen source, but the activity of dihydropyrimidine dehydrogenase was decreased. However, the activities of the enzymes were not regulated by the carbon source (Kim and West, 1991).

Pseudomonas stutzeri was found to be able to reductively catabolize uracil or thymine (Xu and West, 1992). Dihydropyrimidine dehydrogenase had the highest activity when the cells were grown in thymine as the nitrogen source, and the activities of all three enzymes were influenced by the nitrogen source provided to the microorganism. The nicotinamide cofactor specificity for dihydropyrimidine dehydrogenase in *P. stutzeri* was found to be NADH, but activity was observed for the enzyme when NADPH served as the cofactor. The activity when NADPH served as the cofactor was likely due to the presence of pyridine nucleotide transhydrogenase. The second enzyme, dihydropyrimidinase, had the highest activity when dihydrouracil served as the nitrogen source, and the amidohydrolase showed increased activity when dihydrothymine was introduced into the growth media (Xu and West, 1992). When the carbon source was varied between succinate and glucose, it was determined that glucose repressed the activity of dihydropyrimidine dehydrogenase and dihydropyrimidinase, but the variation in carbon source did not affect the activity of *N*-carbamoyl- β -alanine amidohydrolase (Xu and West, 1992).

The reductive degradation of pyrimidines was studied in *P. fluorescens*, and three reductive pathway enzymes were detectable. The first enzyme, dihydropyrimidine

dehydrogenase, was able to function with either nicotinamide cofactor, but the activity was higher when NADPH served as the enzyme cofactor. It is thought that this could be due to pyridine nucleotide transhydrogenase activity. The nitrogen compounds that yielded the highest activity of the reductive pathway enzymes were uracil, thymine, and dihydrothymine (Santiago and West, 1999).

The reductive pathway has not been investigated in *Pseudomonas lemonnieri*. This investigation will concentrate on determining the mechanism of pyrimidine base catabolism in *P. lemonnieri* ATCC 12983. In order to accomplish this, the specific activities of the three reductive pathway enzymes were determined after the microorganism was grown in a variety of nitrogen sources, including uracil, thymine, dihydrouracil, dihydrothymine, β -alanine, β -aminoisobutyric acid, cytosine, and ammonium sulfate. In addition, the carbon source was varied between glucose and succinate. The variation of carbon and nitrogen sources will help to reveal the regulation of the enzymes. Also, the cofactor specificity for dihydropyrimidine dehydrogenase will be determined. Finally, this study investigates the comparison of the reductive pathway enzymes in the parent strain as well as a mutant strain, BS0100.

MATERIALS AND METHODS

Chemicals

Potassium monobasic, potassium dibasic, sodium chloride, sodium citrate, magnesium sulfate heptahydrate, succinate, lysozyme, trichloroacetic acid, magnesium chloride, ethylenediaminetetraacetic acid, Tris, and agar were purchased from the Fisher Company. Glucose, thymine, coomassie brilliant blue, penicillin, *N*-carbamoyl- β -alanine, NADH, and 2-mercaptoethanol were purchased from Sigma. Uracil, dihydrouracil, β -alanine, β -amino-isobutyric acid, ethyl methyl sulfonate, 2-ketoglutarate, antipyrine, diacetylmonoxime, and NADPH were purchased from Acros Organics, a Fisher company. Cytosine was purchased from Lab Guard. Nutrient broth was purchased from Becton-Dickson. Glutamate dehydrogenase was purchased from Roche.

Strain and Growth Media

The microorganism *Pseudomonas lemonnieri* ATCC 12983 was investigated in this study. In order to starve the bacteria of nitrogen and carbon sources, the microorganism was grown in Stanier minimal liquid medium (Stanier, 1947). Stanier's minimal medium consists of KH_2PO_4 (1g/L), K_2HPO_4 (1g/L), NaCl (1g/L), sodium citrate (0.5g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.7g/L), a carbon source (4.0 g/L), and a nitrogen source (2.0 g/L). Glucose or succinate served as the carbon source, and the nitrogen source was varied between uracil, dihydrouracil, beta alanine, thymine, dihydrothymine, beta-amino isobutyric acid, cytosine, and ammonium sulfate.

Preparation of Cell Extracts

To prepare the cells for enzyme assays, the microorganism was transferred from a nutrient agar plate into thirty milliliters of liquid minimal medium with a single carbon and nitrogen source and shaken at 200 revolutions per minute at 30°C. After overnight growth, two milliliters of this batch culture were reinoculated into thirty milliliters of fresh liquid minimal medium with the same carbon and nitrogen source. Three reinoculations were performed for each sample so that an average and standard deviation could be calculated. Growth of the cells in each sample were measured spectrophotometrically at 600nm.

When the bacteria reached mid-exponential phase, the cells were collected by centrifugation at 10,400 x *g* for 20 minutes at 4°C. The cells were washed two times with 0.85% sterile sodium chloride, and then the cell pellets were resuspended in four milliliters of dialysis buffer. The dialysis buffer consisted of 20mM Tris-HCl buffer pH 7.5, 1mM ethylenediaminetetraacetic acid (EDTA), and 1mM 2-mercaptoethanol. Sonication was performed on the suspension at maximum power in thirty second bursts ten times each, for a total time of five minutes each. The sonicated cell extracts were then centrifuged for 30 minutes at 10,900 x *g* at 4°C. The cell-free extracts were each pipetted into dialysis tubing which was then placed in 300mL of dialysis buffer overnight. After dialysis was performed, the cell-free extracts were each assayed for the three pyrimidine catabolic reductive pathway enzymes as well as cytosine deaminase and pyridine nucleotide transhydrogenase.

Enzyme Assays

All enzyme activities were determined from the average of three separate cell-free extracts.

The first enzyme of the pyrimidine reductive pathway, dihydropyrimidine dehydrogenase, was assayed in a one milliliter reaction mixture containing 0.1mM Tris-HCl buffer pH 7.5, 0.2mM cofactor (NADH or NADPH), and cell-free extract. The reaction mixture was incubated at 30°C for five minutes, and then 1mM of the enzyme substrate (uracil or thymine) was added, and water was added to the control mixture. The conversion of NAD(P)H to NAD(P) was followed spectrophotometrically at 340nm for 9.5 minutes (Hunninghake, 1965). The molar absorptivity coefficient for this conversion is $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The activity for this enzyme is expressed as nanomoles of dihydrouracil produced/minute.

In order to determine its activity, the second enzyme of the pyrimidine reductive pathway, dihydropyrimidinase, was assayed colormetrically (West, Shanley, and O'Donovan, 1982). The one milliliter reaction mixture for this assay consisted of 0.1M Tris-HCl buffer pH 7.5, 0.01M MgCl_2 , and cell-free extract. After incubating the reaction mixtures for five minutes at 30°C, the reaction was begun by adding the enzyme substrate dihydrouracil (0.1M), and the reaction was assayed over thirty minutes at 30°C. One milliliter of color mix terminated the reactions throughout the thirty-minute period. The color mix contained one part oxime reagent and two parts antipyrine reagent (West, Shanley, and O'Donovan, 1982). The antipyrine reagent consisted of five grams of antipyrine dissolved in one liter of 50% (v/v) 85% phosphoric acid (West, Shanley, and O'Donovan, 1982). The oxime reagent consisted of 0.80 grams of diacetylmonoxime

dissolved in 5% (v/v) acetic acid (Prescott and Jones, 1969). Once terminated, the reaction tubes were placed on ice until all reactions had been terminated. At this point, all reaction tubes were covered with marbles and placed in a 70°C water bath for 120 minutes under fluorescent light. After two hours, the tubes were cooled to room temperature, and then their absorbencies were measured at 466nm. The absorption coefficient for *N*-carbamoyl- β -alanine (the enzyme product) was experimentally determined from a standard curve based on known NCBA concentration. The activity of this enzyme is expressed as nanomoles of NCBA produced/minute.

The third enzyme of the pyrimidine reductive pathway was assayed by measuring the amount of ammonium ions produced by coupling the last reaction of the reductive pathway with the glutamate dehydrogenase reaction (Tamaki and Mizutani, 1987). The first one milliliter reaction mixture for this assay consisted of 0.1M Tris-HCl buffer pH 7.5, 0.01M MgCl₂, and cell-free extract. The reaction mixtures were incubated for five minutes at 30°C, and then the enzyme substrate *N*-carbamoyl- β -alanine was added and the reaction was assayed over a 30-minute period. For this assay, 50% (w/v) trichloroacetic acid terminated the reaction. The protein precipitate was removed by low speed centrifugation, and 0.5 milliliters of the supernatant was added to a second reaction mixture that contained 0.2mL of 2M KHCO₃, 0.2mL of 0.5M Tris-HCl buffer pH 8.0, 0.1mL of 0.1M 2-ketoglutarate, and 0.02mL of 12mM NADH. An initial absorbance reading was taken at 365nm as the control for each reaction mixture, and then 12 units/mL of glutamate dehydrogenase was added to each mixture. These mixtures were allowed to sit for sixty minutes at room temperature, and then their final absorbencies were measured at 365nm. The amination of ketoglutarate to glutamate by incorporation

of the ammonia end product was determined by the difference in absorbencies between the mixtures at 0 minutes and 60 minutes (Tamaki and Mizutani, 1987). The activity of this enzyme is expressed as nanomoles of NCBA utilized/minute.

The enzyme cytosine deaminase, which converts cytosine to uracil, was assayed spectrophotometrically at 285nm. The one milliliter reaction mixture for the cytosine deaminase assay consisted of 0.05M Tris-HCl buffer pH 7.3 and cell free extracts. The enzyme substrate, 0.5mM cytosine, was added, and water served as the control. The absorbance of the reaction mixture was measured at 30°C for 9.5 minutes, and the molar absorptivity coefficient for the conversion of cytosine to uracil is $1.038 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (West, 1992). The activity for this enzyme is expressed as nanomoles of dihydrouracil produced/minute.

The enzyme pyridine nucleotide transhydrogenase, which translocates hydrogen, was assayed spectrophotometrically at 400nm. The one milliliter reaction mixture for this assay consisted of 0.1mM reduced NADP, 0.05M Tris-HCl buffer pH 7.5, and cell-free extract. The enzyme substrate, 0.1mM thionicotinamide adenine dinucleotide (tNAD⁺), was added, and water served as the control. The absorbance of the reaction mixture was measured at 30°C for 9.5 minutes, and the molar absorptivity coefficient for the conversion between the oxidized and reduced form of tNAD is $11.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (French et. al., 1997).

Mutagenesis

The parent strain *Pseudomonas lemonnieri* ATCC 12983 was mutated using the mutagen ethyl methyl sulfonate (Watson and Holloway, 1976; Watrin et. al., 1999).

First, the parent strain was grown to mid-exponential phase in nutrient broth. This culture was then diluted in a 10:1 ratio with the mutagen EMS. After being gently mixed, the dilution was left to sit for sixty minutes. One milliliter of the EMS-treated bacteria was grown in nutrient broth to mid-exponential phase. The culture was then collected by low speed centrifugation and diluted with 0.85% sterile saline and inoculated onto 200 μ g/mL penicillin plates. A colony from this antibiotic plate was isolated and grown in nutrient broth. In order to ensure mutagenesis had taken place, the parent strain was also grown on 200 μ g/mL penicillin plates.

Protein Assay

In order to determine the specific activity of the enzymes of the pyrimidine reductive pathway, the amount of protein in each cell-free extract was determined using the Bradford Method and lysozyme as the standard protein (Bradford, 1976). The dye that was used to bind non-specifically to the protein consisted of 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. The cell-free extracts were diluted in a 1:10 ratio with water, and then 0.1mL of the diluted protein was mixed with 5.0mL of the dye mixture. In addition, a standard curve using known concentrations of lysozyme plotted against their absorbencies at 595nm was created and used to determine the amount of protein in each dilution.

RESULTS

The primary goal of this research project was to determine whether or not the reductive pathway exists in *Pseudomonas lemonnieri* ATCC 12983 and the mutant strain BS0100. In order to determine if the species was able to reductively degrade pyrimidines, the specific activities of the three reductive pathway enzymes, namely, dihydropyrimidine dehydrogenase, dihydropyrimidinase, and *N*-carbamoyl- β -alanine amidohydrolase, were measured. In addition, the specific activity of the enzyme pyridine nucleotide transhydrogenase was measured to determine if there was evidence for cofactor specificity for dihydropyrimidine dehydrogenase. Also, the specific activity of cytosine deaminase was measured in both the parent and mutant strains in order to determine if *P. lemonnieri* was able to degrade cytosine into uracil.

Pseudomonas lemonnieri ATCC 12983 was grown in liquid minimal media containing glucose as the carbon source, and in order to determine control values for the specific activities of the reductive pathway enzymes, a source of nitrogen was not provided in the media. The cell free extracts were assayed for dihydropyrimidine dehydrogenase, dihydropyrimidinase, and *N*-carbamoyl- β -alanine amidohydrolase. The results of these assays showed low specific activities for all three enzymes (Table 1). Dihydropyrimidine dehydrogenase showed the lowest specific activity, while *N*-carbamoyl- β -alanine amidohydrolase showed the highest.

The reductive pathway enzymes were then assayed in ATCC 12983 cells grown in a liquid minimal media containing glucose as the carbon source and uracil as the nitrogen source. These experiments showed that the reductive pathway enzymes are active in the parent strain (Table 2). Dihydropyrimidine dehydrogenase again showed the

Table 1: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.10 [0.01]
Dihydropyrimidinase	0.18 [0.03]
N-carbamoyl- β -alanine amidohydrolase	0.50 [0.01]

The cells were grown 0.4% carbon source and no source of nitrogen. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

Table 2: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and uracil as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	1.23 [0.03]**
Dihydropyrimidinase	10.9 [0.80]**
N-carbamoyl- β -alanine amidohydrolase	4.14 [0.20]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from control grown with no nitrogen source at $p < 0.01$

lowest specific activity, but dihydropyrimidinase showed the highest activity level. As compared to the parent strain control (no nitrogen source), dihydropyrimidine dehydrogenase activity was approximately twelve times higher in the cells grown with uracil, and dihydropyrimidinase activity was approximately sixty-one times higher in the uracil-grown cells. *N*-carbamoyl- β -alanine amidohydrolase activity was approximately eight times higher in the cells grown with uracil. These results reveal that the presence of the initial substrate of the reductive pathway, uracil, in the media greatly enhances all three enzyme activities.

Two nicotinamide cofactors were studied as possible cofactors for dihydropyrimidine dehydrogenase. The first cofactor that was studied was the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Table 3). When uracil was used as the substrate for the reaction during the assay with NADPH serving as the cofactor, its activity was substantially higher than when thymine was used as the substrate. However, when a second cofactor, the reduced form of nicotinamide adenine dinucleotide (NADH), was studied, the specific activities when uracil or thymine was used as the substrate were only slightly different (Table 4). Independent of the substrate used, the specific activity of dihydropyrimidine dehydrogenase was significantly higher when NADPH served as the cofactor. In order to further investigate the specificity of cofactor for dihydropyrimidine dehydrogenase, an assay was performed for pyridine nucleotide transhydrogenase. The results of this experiment show that the enzyme is active in the bacterium (Table 5).

The reductive pathway enzymes were also assayed in ATCC 12983 cells grown in liquid minimal media containing glucose as the carbon source and dihydrouracil as the

Table 3: Dihydropyrimidine dehydrogenase specific activities in *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and uracil as the nitrogen source when NADPH served as the cofactor.

Enzyme Substrate	Specific Activity
Uracil	1.23 [0.03]
Thymine	0.74 [0.04]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

Table 4: Dihydropyrimidine dehydrogenase specific activities in *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and uracil as the nitrogen source when NADH served as the cofactor.

Enzyme Substrate	Specific Activity
Uracil	0.13 [0.02]
Thymine	0.10 [0.01]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

Table 5: Pyridine nucleotide transhydrogenase specific activity for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium.

Carbon Source	Nitrogen Source	Specific Activity
Glucose	Uracil	0.31 [0.05]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. The value is the mean of three separate trials [std dev].

nitrogen source (Table 6). Following the same trend as the parent cells grown in uracil media, dihydropyrimidine dehydrogenase showed the lowest specific activity while dihydropyrimidinase showed the highest specific activity. However, all three enzymes in the dihydrouracil-grown cells show lower activity levels than those in the uracil-grown cells. Even though all the enzymes show decreased activity levels when grown in dihydrouracil media, they are still higher than when the cells were grown without a nitrogen source.

In the following assays, the ATCC 12983 cells were grown in liquid minimal media containing glucose as the carbon source and β -alanine as the nitrogen source. When the end product of the uracil reductive pathway was provided as the nitrogen source for the cells, the enzyme activities did not follow the same trend as with uracil and dihydrouracil (Table 7). The results of these experiments revealed the same trend as seen when the cells were grown without a nitrogen source. Dihydropyrimidine dehydrogenase showed the lowest specific activity while *N*-carbamoyl- β -alanine amidohydrolase showed the highest. However, while the activities of dihydropyrimidine dehydrogenase and dihydropyrimidinase were only slightly elevated when grown on β -alanine, the specific activity of *N*-carbamoyl- β -alanine amidohydrolase was significantly increased by more than fourteen times as compared to the cells grown with no nitrogen source. When compared to the parent strain that was grown on uracil, the activities of dihydropyrimidine dehydrogenase and dihydropyrimidinase are significantly decreased in the β -alanine grown cells, but the *N*-carbamoyl- β -alanine amidohydrolase specific activity is nearly doubled in the β -alanine grown cells.

Table 6: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and dihydrouracil as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.44 [0.03]**
Dihydropyrimidinase	3.30 [0.24]**
N-carbamoyl- β -alanine amidohydrolase	0.60 [0.02]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from control grown with no nitrogen source at $p < 0.01$

Table 7: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and β -alanine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.29 [0.02]**
Dihydropyrimidinase	0.76 [0.01]**
N-carbamoyl- β -alanine amidohydrolase	7.26 [0.63]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from control grown with no nitrogen source at $p < 0.01$

The reductive pathway enzymes were also assayed in extracts from ATCC 12983 cells grown in liquid minimal media containing glucose as the carbon source and thymine as the nitrogen source. The results from these experiments show that the reductive pathway enzymes are functioning in the cells (Table 8). Dihydropyrimidine dehydrogenase has the lowest activity, and dihydropyrimidinase has the highest activity. This is the same trend as seen in the cells grown with uracil as the source of nitrogen. However, for the thymine-grown cells, all three enzymes had significantly lower specific activities than when the cells were grown in uracil. When compared to the control (no nitrogen source) specific activities, dihydropyrimidinase and *N*-carbamoyl- β -alanine amidohydrolase specific activities for the thymine-grown cells are both higher than when no nitrogen source was provided. However, the activity of dihydropyrimidine dehydrogenase is lower in the thymine-grown cells than in the cells provided with no nitrogen source. This does not parallel the trend seen between the uracil-grown cells and the control cells.

The reductive pathway enzymes were also assayed for ATCC 12983 cells grown in liquid minimal media containing glucose as the carbon source and dihydrothymine as the nitrogen source (Table 9). Dihydropyrimidine dehydrogenase showed the lowest specific activity, while dihydropyrimidinase showed the highest activity level. The specific activities of dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase are similar between the thymine-grown cells and the dihydrothymine-grown cells, but the level of dihydropyrimidinase is significantly decreased in the dihydrothymine-grown cells. When compared to the cells grown in dihydrouracil, the

Table 8: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and thymine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.06 [.003]**
Dihydropyrimidinase	2.36 [0.32]**
N-carbamoyl- β -alanine amidohydrolase	0.74 [0.03]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from control grown with no nitrogen source at $p < 0.01$

Table 9: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and dihydrothymine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.08 [0.01]*
Dihydropyrimidinase	0.81 [0.04]**
N-carbamoyl- β -alanine amidohydrolase	0.65 [0.04]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

* Significantly different from control grown with no nitrogen source at $p < 0.05$

** Significantly different from control grown with no nitrogen source at $p < 0.01$

dihydrothymine-grown cells showed significantly lower activities for dihydropyrimidine dehydrogenase and dihydropyrimidinase.

When the reductive pathway enzymes were assayed for ATCC 12983 cells grown in liquid minimal media with glucose as the carbon source and β -aminoisobutyric acid as the nitrogen source (Table 10), the same trend was seen as when the cells were grown with β -alanine as the nitrogen source. Dihydropyrimidine dehydrogenase was shown to have the lowest activity, and *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity. Dihydropyrimidine dehydrogenase and dihydropyrimidinase both showed lower activity levels in the β -aminoisobutyric-grown cells when compared to the β -alanine-grown cells. However, the activity of *N*-carbamoyl- β -alanine amidohydrolase was higher in the β -aminoisobutyric-grown cells than in the β -alanine-grown cells. When compared to thymine-grown cells, the activities of dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase are higher in the β -aminoisobutyric-grown cells, but the dihydropyrimidinase activity is higher for thymine-grown cells. All three enzyme activities are higher in the β -aminoisobutyric-grown cells when compared to the control cells that were not provided with any nitrogen source.

The reductive pathway enzymes were also assayed for ATCC12983 cells grown in liquid minimal media containing glucose as the carbon source and ammonium sulfate as the nitrogen source (Table 11). Dihydropyrimidine dehydrogenase showed the lowest specific activity, and *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity level. This follows the same trend as the control (no nitrogen source) cells. However, while dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase activities are higher in the no nitrogen source cells, dihydropyrimidinase activity is higher

Table 10: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and β -amino- isobutyric acid as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.13 [0.01]*
Dihydropyrimidinase	0.44 [0.04]**
N-carbamoyl- β -alanine amidohydrolase	18.3 [1.20]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

* Significantly different from control grown with no nitrogen source at $p < 0.05$

** Significantly different from control grown with no nitrogen source at $p < 0.01$

Table 11: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and ammonium sulfate as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.06 [0.01]**
Dihydropyrimidinase	0.24 [0.02]*
N-carbamoyl- β -alanine amidohydrolase	0.45 [0.02]*

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

* Significantly different from control grown with no nitrogen source at $p < 0.05$

** Significantly different from control grown with no nitrogen source at $p < 0.01$

in the ammonium sulfate-grown cells. When compared to uracil-grown cells, all three enzyme activities are lower in the ammonium sulfate-grown cells. However, when compared to the other initial substrate of the pathway, thymine, dihydropyrimidine dehydrogenase activity is higher in the ammonium sulfate-grown cells.

In order to determine if *P. lemonnieri* could degrade cytosine and then utilize the reductive pathway, the reductive pathway enzymes were assayed for ATCC 12983 cells grown in liquid minimal media containing glucose as the carbon source and cytosine as the nitrogen source (Table 12). From these results, it is evident that the reductive pathway is operating in these cells. Like the control and the ammonium sulfate-grown cells, the lowest activity is shown to be dihydropyrimidine dehydrogenase, and the highest activity is for *N*-carbamoyl- β -alanine amidohydrolase. However, all three enzyme activities for the cytosine-grown cells are higher than both the control activities and the activities for the ammonium sulfate-grown cells. When compared to uracil, dihydropyrimidine dehydrogenase and dihydropyrimidinase activities were both lower in the cytosine-grown cells, but *N*-carbamoyl- β -alanine amidohydrolase was lower in the uracil-grown cells.

The reductive pathway enzymes were also assayed in ATCC 12983 cells grown in liquid minimal media containing succinate as the carbon source and uracil as the nitrogen source (Table 13). Following the same trend as the cells grown in the glucose-uracil media, dihydropyrimidine dehydrogenase showed the lowest activity, while dihydropyrimidinase showed the highest activity. However, all three enzymes showed lower specific activity when assayed from the succinate-uracil grown cells when compared to the glucose-uracil grown cells. Dihydropyrimidine dehydrogenase was

Table 12: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing glucose as the carbon source and cytosine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.11 [0.02]
Dihydropyrimidinase	1.15 [0.16]**
N-carbamoyl- β -alanine amidohydrolase	5.95 [1.12]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from control grown with no nitrogen source at $p < 0.01$

Table 13: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing succinate as the carbon source and uracil as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.09 [0.01]**
Dihydropyrimidinase	2.06 [0.04]**
N-carbamoyl- β -alanine amidohydrolase	1.21 [0.03]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from control grown in glucose/uracil minimal medium at $p < 0.01$

assayed with two different reduced nicotinamide cofactors (NADH or NADPH) and with the two different enzyme substrates (uracil or thymine). When NADPH served as the cofactor, dihydropyrimidine dehydrogenase showed highest activity when thymine was used as the substrate (Table 14). However, when NADH served as the cofactor, there was no difference in the enzyme activity when either uracil or thymine was used as the substrate (Table 15). The combination that yielded the highest specific activity for dihydropyrimidinase was when NADPH was utilized as the cofactor and thymine served as the substrate. When pyridine nucleotide transhydrogenase was assayed in the succinate-uracil grown cells, activity was present (Table 16). However, it was significantly lower than when the enzyme was assayed for cells grown in glucose-uracil media.

The three reductive pathway enzymes also showed activity in ATCC 12983 cells that were grown in liquid minimal media containing succinate as the carbon source and thymine as the nitrogen source (Table 17). Dihydropyrimidine dehydrogenase showed the lowest activity, and *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity. When compared to the succinate-uracil and glucose-thymine grown cells, dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase activities were highest in the succinate-thymine grown cells.

The reductive pathway enzymes were also assayed in mutant strain BS0100 cells grown in liquid minimal media containing glucose as the carbon source and uracil as the nitrogen source (Table 18). A new trend was seen in the activities of these enzymes. Dihydropyrimidinase showed the lowest activity, and *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity. When compared to the ATCC 12983 cells

Table 14: Dihydropyrimidine dehydrogenase specific activities in *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing succinate as the carbon source and uracil as the nitrogen source when NADPH served as the cofactor.

Enzyme Substrate	Specific Activity
Uracil	0.09 [0.01]
Thymine	0.20 [0.01]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

Table 15: Dihydropyrimidine dehydrogenase specific activities in *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing succinate as the carbon source and uracil as the nitrogen source when NADH served as the cofactor.

Enzyme Substrate	Specific Activity
Uracil	0.13 [0.01]
Thymine	0.13 [0.01]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

Table 16: Pyridine nucleotide transhydrogenase specific activity for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium.

Carbon Source	Nitrogen Source	Specific Activity
Succinate	Uracil	0.31 [0.05]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. The value is the mean of three separate trials [std dev].

Table 17: Pyrimidine reductive pathway enzyme activities for *Pseudomonas lemonnieri* ATCC 12983 cells grown in minimal medium containing succinate as the carbon source and thymine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.60 [0.03]**
Dihydropyrimidinase	1.32 [0.30]*
N-carbamoyl- β -alanine amidohydrolase	11.7 [0.40]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

* Significantly different from control grown in glucose/thymine minimal medium at $p < 0.05$

** Significantly different from control grown in glucose/thymine minimal medium at $p < 0.01$

Table 18: Pyrimidine reductive pathway enzyme activities for mutant strain BS0100 cells grown in minimal medium containing glucose as the carbon source and uracil as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.38 [0.02]**
Dihydropyrimidinase	0.25 [0.05]**
N-carbamoyl- β -alanine amidohydrolase	9.56 [0.84]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from parent cells grown in glucose/uracil minimal medium at $p < 0.01$

that were grown in a glucose-uracil media, the parent cells showed the higher activity with respect to dihydropyrimidine dehydrogenase and dihydropyrimidinase, but the mutant strain *N*-carbamoyl- β -alanine amidohydrolase showed significantly higher activity than the parent strain.

The reductive pathway enzymes were also assayed in BS0100 cells grown in liquid minimal media containing glucose as the carbon source and thymine as the nitrogen source (Table 19). Dihydropyrimidine dehydrogenase showed the lowest specific activity while *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity. When compared to the mutant cells grown in glucose-uracil media, dihydropyrimidine dehydrogenase showed lower activity in the glucose-thymine media, but the other two reductive pathway enzyme activities were increased in the glucose-thymine grown mutant cells. Dihydropyrimidinase activity was higher in the parent cells grown in glucose-thymine media, but dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase activities were higher in the mutant cells grown in glucose-thymine media.

The reductive pathway enzymes were also assayed for BS0100 cells grown in liquid minimal media containing succinate as the carbon source and uracil as the nitrogen source (Table 20). Dihydropyrimidine dehydrogenase showed the lowest specific activity, and *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity level. Dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase activities were higher in the mutant cells grown in succinate-uracil media when compared to the parent cells grown in the same media. However, when compared to the mutant cells

Table 19: Pyrimidine reductive pathway enzyme activities for mutant strain BS0100 cells grown in minimal medium containing glucose as the carbon source and thymine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.18 [0.02]**
Dihydropyrimidinase	0.31 [0.02]**
N-carbamoyl- β -alanine amidohydrolase	14.5 [0.78]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

** Significantly different from parent cells grown in glucose/thymine minimal medium at $p < 0.01$

Table 20: Pyrimidine reductive pathway enzyme activities for mutant strain BS0100 cells grown in minimal medium containing succinate as the carbon source and uracil as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.17 [0.02]**
Dihydropyrimidinase	1.69 [0.22]*
N-carbamoyl- β -alanine amidohydrolase	5.91 [0.19]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

* Significantly different from parent cells grown in succinate/uracil minimal medium at $p < 0.05$

** Significantly different from parent cells grown in succinate/uracil minimal medium at $p < 0.01$

grown in glucose-uracil media, dihydropyrimidinase was the only enzyme that showed higher activity in the mutant cells grown in succinate-uracil media.

The reductive pathway enzymes were also assayed for BS0100 cells grown in liquid minimal media containing succinate as the carbon source and thymine as the nitrogen source (Table 21). Like the mutant cells grown in succinate-uracil media, *N*-carbamoyl- β -alanine amidohydrolase showed the highest activity, and dihydropyrimidine dehydrogenase showed the lowest activity. Also, both dihydropyrimidine dehydrogenase and *N*-carbamoyl- β -alanine amidohydrolase showed higher activities in the mutant cells grown in succinate-thymine media when compared to the mutant cells grown in succinate-uracil media. All three enzyme activities were higher for the mutant cells grown in succinate-thymine media when compared to the mutant cells grown in glucose-thymine media. When compared to the parent cells grown in succinate-thymine media, *N*-carbamoyl- β -alanine amidohydrolase was the only enzyme that showed higher activity in the mutant cells grown in succinate-thymine media.

The specific activity of cytosine deaminase, which converts cytosine to uracil, was measured in cells grown in a variety of different liquid minimal medias (Table 22). When ATCC 12983 cells were grown in glucose-uracil media, there was no detectable cytosine deaminase activity. However, when the cells were grown in glucose-cytosine media, the activity was significantly increased. Again, when the ATCC 12983 cells were grown in succinate-uracil media, there was no cytosine deaminase activity, but when cytosine was substituted as the nitrogen source, the activity significantly increased. The cytosine deaminase activity is much higher in the succinate-cytosine grown parent cells when compared to the glucose-cytosine grown parent cells. When BS0100 cells were

Table 21: Pyrimidine reductive pathway enzyme activities for mutant strain BS0100 cells grown in minimal medium containing succinate as the carbon source and thymine as the nitrogen source.

Enzyme	Specific Activity
Dihydropyrimidine dehydrogenase	0.20 [.004]**
Dihydropyrimidinase	0.90 [0.08]*
N-carbamoyl- β -alanine amidohydrolase	17.3 [1.65]**

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

* Significantly different from parent cells grown in succinate/thymine minimal medium at $p < 0.05$

** Significantly different from parent cells grown in succinate/thymine minimal medium at $p < 0.01$

Table 22: Cytosine deaminase specific activities for *Pseudomonas lemonnieri* cells grown in minimal medium.

Cells	Carbon/Nitrogen Sources	Specific Activity
ATCC 12983	Glucose/Uracil	0.00 [0.00]
ATCC 12983	Glucose/Cytosine	17.7 [1.64]
ATCC 12983	Succinate/Uracil	0.00 [0.00]
ATCC 12983	Succinate/Cytosine	43.1 [5.80]
BS0100	Glucose/Uracil	0.00 [0.00]
BS0100	Glucose/Cytosine	114. [8.60]
BS0100	Succinate/Uracil	0.00 [0.00]
BS0100	Succinate/Cytosine	75.7 [8.00]

The cells were grown in 0.2% nitrogen source and 0.4% carbon source. Specific activities are measured as nanomoles/minute/milligram of protein at 30°C. Each value is the mean of three separate trials [std dev].

grown in glucose-uracil media, there was again no detectable cytosine deaminase activity, but when the media was glucose-cytosine, the activity dramatically increased. There was also a significant increase in activity in the mutant cells when compared to the parent cells in the glucose-cytosine media. When the BS0100 cells were grown in succinate-uracil media, there was no cytosine deaminase activity. However, there was significantly increased activity when the media was composed of succinate and cytosine. When comparing the mutant cells grown in succinate-cytosine and glucose-cytosine medias, the highest cytosine deaminase activity was shown when glucose served as the carbon source. The cytosine deaminase activity in the mutant cells grown in succinate-cytosine media was significantly higher than the activity in the parent cells grown in the same media.

DISCUSSION

The purpose of this project was to determine if *P. lemonnieri* ATCC 12983 cells utilized the reductive pathway for the catabolism of pyrimidines. Based on the results from this investigation, it was determined that *P. lemonnieri* could reductively degrade pyrimidines.

The highest dihydropyrimidine dehydrogenase activity was observed when *P. lemonnieri* was grown in media containing glucose as the carbon source and uracil as the nitrogen source. This activity was 12.3 times greater than when there was no nitrogen source and twenty times greater than in the ammonium sulfate-repressed cells. This is expected since uracil is the initial substrate of the reductive pathway. There was also evidence that in this media, NADPH was favored as the nicotinamide cofactor. However, since NADH in the reaction mixture also yielded activity, it is likely that the organism can utilize either of the two nicotinamide cofactors. This was also seen for *P. stutzeri* (Xu and West, 1992) and *P. fluorescens* (Santiago and West, 1999). The specificity for NADPH can be explained by the presence of activity of pyridine nucleotide transhydrogenase, which catalyzes the reduction of NAD^+ to NADH by way of direct hydrogen transfer from NADPH (Kaplan et. al., 1952; Colowick et. al., 1952). The enzyme was found to be inhibited by NADP^+ in *P. fluorescens*, which effectively reduced the production of NADH (French et. al., 1997).

The lowest dihydropyrimidine dehydrogenase activity for the ATCC 12983 cells was observed when glucose was the carbon source and thymine was the nitrogen source. This activity was equivalent to the ammonium sulfate-repressed cells. All three reductive

pathway enzymes were greatly decreased in the thymine-grown cells when compared to uracil-grown cells. This could be due to a lack of transport mechanism for thymine to enter the cell. Additionally, the first enzyme may not be specific for thymine, which could inhibit the entire pathway.

Dihydropyrimidinase showed the highest activity when the *P. lemonnieri* ATCC 12983 cells were grown in media with glucose serving as the carbon source and uracil as the nitrogen source. This activity was sixty times greater than that observed when there was no nitrogen source present and forty-five times greater than in the ammonium sulfate-repressed cells. *Pseudomonas aeruginosa* was also reported to have highest dihydropyrimidinase activity in glucose-uracil grown cells (Kim and West, 1991).

The final reductive pathway enzyme, *N*-carbamoyl- β -alanine amidohydrolase, was found to have the highest activity in *P. lemonnieri* ATCC 12983 cells grown in media containing glucose as the carbon source and β -aminoisobutyric acid as the nitrogen source. This is thirty-six times greater than the cells grown without a nitrogen source and forty-one times greater than when grown in ammonium sulfate media. This is of great interest because this reaction has been reported to be irreversible (O'Donovan and Neuhard, 1970), and therefore the end product of the reaction would not be expected to yield the highest activity. In other pseudomonads, dihydrouracil media yielded the greatest amidohydrolase activity (Kim and West, 1991; Xu and West, 1992).

In addition to the parent strain, *P. lemonnieri* ATCC 12983, a mutant strain, BS0100, was isolated. This mutant strain was found to be penicillin resistant, and in order to determine if the reductive pathway enzymes had been affected in the mutagenesis, the enzymes were assayed for this strain grown in a variety of minimal

media. Due to the activity of the enzymes, it could be concluded that the reductive pathway is operating in the mutant strain, BS0100. Based on the results of these assays, it was determined that the reductive pathway enzymes were likely altered during the mutation process. In the mutant cells, dihydropyrimidinase activity is significantly lower than in the parent strain. Ethylmethyl sulfonate, which causes methylation, could have altered the genetic makeup of the cells to produce a less efficient enzyme. Additionally, the mutagenesis could have altered the signaling method within the cell that communicates to the organism that it needs to produce the enzyme. The activity of the amidohydrolase enzyme increased in the mutant cells.

Cytosine deaminase activity was nonexistent in cells grown in media not containing cytosine, but it was very active in cells that were grown with a cytosine-rich media. This means it is likely that cytosine deaminase production in the cell depends on the presence of cytosine. This hypothesis is supported in earlier studies in *P. fluorescens* and *P. putida* where cytosine deaminase activity was seen to be dependent on the nitrogen source in the media (Chu and West, 1990). When cytosine was introduced into the media in these earlier studies, the cytosine deaminase activity increased when compared to their control activities. The reason for this control is likely energy conservation. The cytosine deaminase activity was significantly increased in the mutant cells grown in cytosine media, which means that the mutation also affected the workings of this enzyme. It is possible that transport of cytosine into the cell was increased by increased permeability or production of necessary transport proteins. Also, the increase in activity could mean that enzyme efficiency was affected by alteration of signaling patterns, or the actual structure of the enzyme could have been changed.

In summary, the reductive pathway was found to be operating in *P. lemonnieri* ATCC 12983 cells as well as the mutant strain BS0100. The enzymes were altered by mutagenesis, which affected their activities within the cell. Nitrogen source variation as well as carbon source variation also greatly affected the specific activities of the reductive pathway enzymes. This investigation produced similar results to those obtained in earlier studies of *Pseudomonas* species. The results from this study are important taxonomically because now this species could be classified based on the reductive pathway enzyme activity within the cells. Since a likely nicotinamide cofactor specificity exists and the two *P. lemonnieri* strains are able to utilize pyrimidines for growth, the species would likely fall under the same DNA homology group as *P. fluorescens*. This is not surprising since *P. lemonnieri* was at one time classified as a biotype of *P. fluorescens*.

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