# Optimizing Atrazine Catabolism in *Pseudomonas* sp. strain ADP

by

Robert Anderson

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Paul Kirchman, and has been approved by the members of her/his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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# Abstract


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Atrazine is a popular herbicide used in over 80 countries to inhibit the growth of broadleaf and grassy weeds. Atrazine is a common pollutant in soil and groundwater, and high concentrations of atrazine cause developmental defects in fish, amphibians, and birds. The bacteria *Pseudomonas* sp. strain ADP (P.ADP) uses atrazine as a nitrogen source by transforming atrazine to ammonia through a number of enzymatic reactions. In this project I measured the growth and atrazine degradation of P.ADP in media with atrazine as the sole nitrogen source. A mutant strain isolated after mutagenesis with UV light showed faster growth and reached higher densities than the control strain. A series of mating experiments were performed to determine whether the growth mutation was on the atrazine degrading plasmid or in the chromosome. The limitations and potential of P.ADP for atrazine degradation are discussed.

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# Introduction

The herbicide atrazine (2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine) is one of the most frequently applied agricultural chemicals in the world. 80 million pounds of atrazine are applied each year in the United States and it is the most common pesticide contaminant in North American ground and surface water (Boyd, 2000). Atrazine levels in groundwater often exceed EPA recommended limit of 3ppm (Boyd, 2000), and the widespread release of this herbicide is believed to pose a significant danger to local ecosystems (Biglione et al., 2008). In plants, atrazine blocks electron flow in photosystem II of photosynthesis, inhibiting production of plastoquinol (Berg et al., 2007, p. 560). In vertebrates, however, various studies suggest atrazine interferes with both individual development and hormone balance. Studies with frogs have shown that concentrations of 1 ppb atrazine led to demasculinization of males, with male frogs having low sperm count and incomplete maturation of male voice boxes (Hayes et al., 2002). Male Xenopus frogs in the presence of 2.5ppb atrazine were even shown to develop as hermaphrodites, or as fully functional phenotypic females (Hayes et al., 2010). Part of the global decline in amphibian populations may due to atrazine's effect on amphibian endocrine systems. Atrazine exposure may also have detrimental effects on fish and bird populations (Hayes et al., 2010). Exposure in humans has been linked to higher incidences of stomach cancer (van Leeuwen et al., 1999), and has also been shown to promote aromatase activity by altering cAMP levels in the body (Sanderson et al., 2000; Roberge et al., 2004).

Because atrazine is present in high concentrations in soils and groundwater, a cheap and efficient method for removing atrazine is necessary. Chemical removal techniques are costly to maintain and, in the case of thermal or catalytic oxidation reactions, toxic byproducts may be formed (Dua et al., 2002). Bioremediation, the breakdown of harmful pollutants by microorganisms, is an attractive alternative to physical or chemical removal of atrazine. Bioremediation is cheaper than physiochemical treatments and can be performed on site in a complex mixture of contaminants. In addition, modern molecular biology and biotechnology allow researchers to create transgenic organisms with the ability to degrade many soil contaminants simultaneously (Pieper and Reineke, 2000).

Competition for resources in low nutrient environments creates high selective pressure for the evolution of novel degradative pathways in soil bacteria. Synthetic organic compounds (xenobiotics) are often metabolized by soil bacteria within decades after first introduction in the environment (Seffernick & Wackett, 2001). Halogenated aromatic hydrocarbons, such as atrazine, are particularly persistent and are difficult to degrade by microorganisms (Dua et al., 2002); however, catabolic degradation of atrazine has been identified in soil bacteria in many different regions. These degradation pathways share a high level of sequence homology and likely arose in a few areas and spread globally by horizontal gene expansion. Bacteria obtain novel degradation pathways by using other similar catabolic mechanisms as starting points for evolution, and the atrazine degrading plasmid likely arose from genetic recombination of the melamine degrading plasmid (Seffernick and Wackett, 2001). Melamine is a similarly structured substrate and many of the genes on the atrazine degrading plasmid share sequence homology to the melamine degrading plasmid.

The degradation pathway of the species Pseudomonas sp. strain ADP is the best characterized pathway of atrazine degradation. Complete mineralization of atrazine by Pseudomonas sp. ADP occurs through a series of six enzymatic reactions. Atrazine is transformed to hydroxyatrazine by hydrolytic dehalogenation of the triazine ring, followed by hydrolytic deamination of the two amine ring substituents, to yield cyanuric acid (Figure 1). Genes atzA, atzB and atzC encode for the enzymes in these reactions, and cyanuric acid is further degraded into biuret and ammonia by enzymes encoded in genes atzD atzE and atzF.

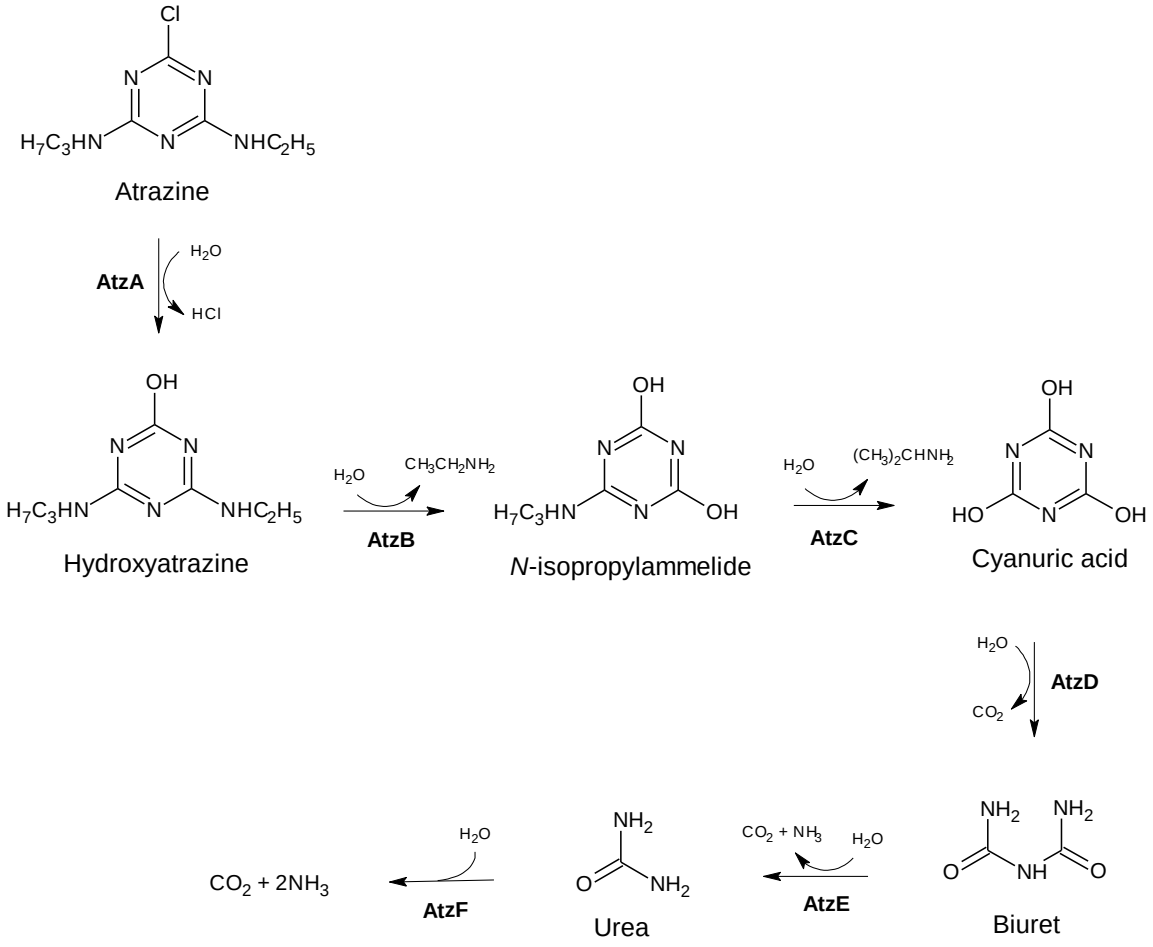


Figure 1 – Atrazine degradation pathway in *Pseudomonas* sp. strain ADP.

All the genes for atrazine degradation are encoded on the self transmissible plasmid pADP-1 (Figure 2). The coding regions for the first three reactions (atzA, atzB, atzC) are spread out on the plasmid and separated by transposable elements, indicating multiple independent gene transfer events (Martinez et al., 2001). Gene sequences for atzA, atzB, and atzC are highly conserved across species, suggesting a recent evolution and propagation of catabolic genes. Considering atrazine was not present in the environment before its industrial synthesis 43 years ago (Jablonowski et al., 2008), a recent evolution of atrazine catabolism is most likely. The genes atzD, atzE, and atzF, coding for the breakdown of cyanuric acid, are localized next to each other on the plasmid, and are encoded on a single mRNA. Cyanuric acid is a common intermediate in the breakdown of triazines, and melamine and ammeline are degraded to cyanuric acid in nitrogen metabolism of other species prior to cleavage of the triazine ring (de Souza et al., 1996). The atzDEF genes are believed to be regulated by the gene atzR in an operon-like manner (González et al., 2005).

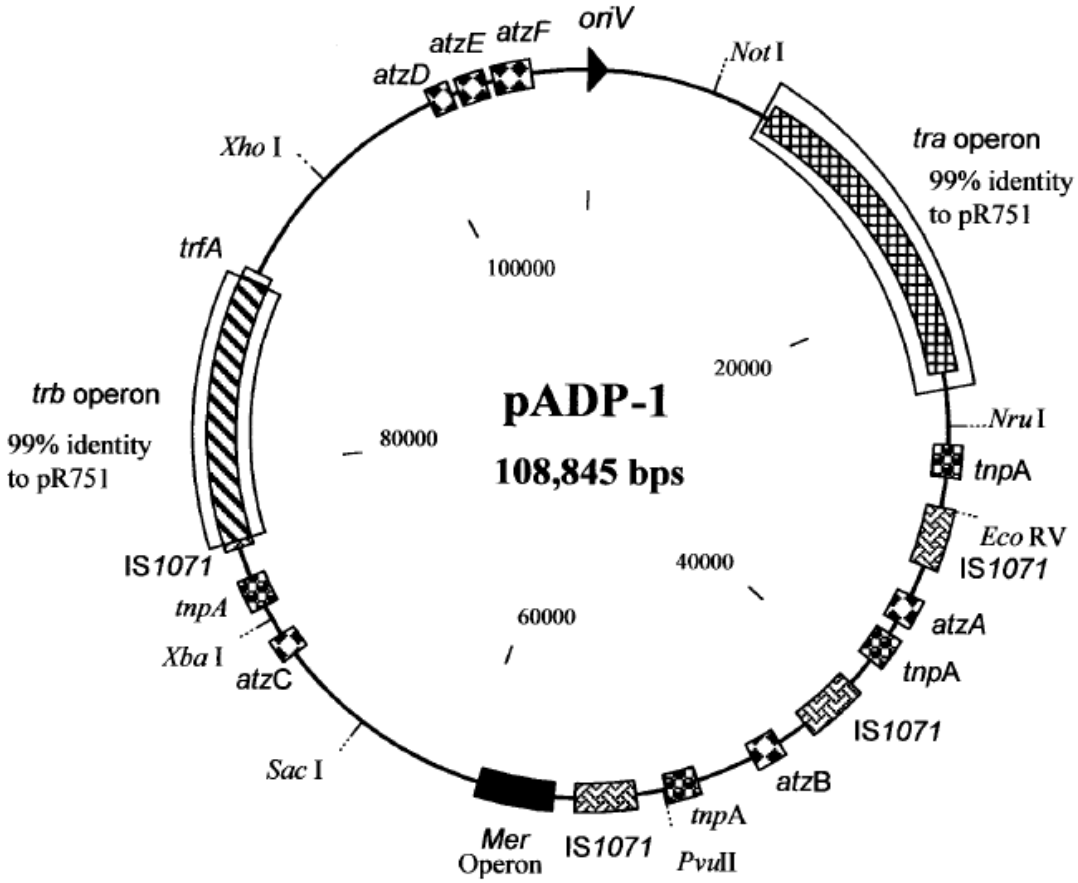


Figure 2 - Circular Map of the atrazine degrading plasmid (p.ADP 1) from *Pseudomonas* sp. strain ADP. Taken from Martinez et al., 2001.

The potential for degradation of a particular compound by bioremediation is limited by a variety of factors. Though the environment may select for bacteria with the desired characteristics, natural evolution is too slow to prevent the environmental and public health consequences of increasing atrazine concentrations. Biotechnology can speed up the selective process and design microorganisms that are highly adapted to degradation of persistent organic pollutants. In this project, we investigate the potential of Pseudomonas sp. ADP for atrazine bioremediation, and, using genetic manipulation, we demonstrate a procedure for optimizing bacteria for bioremediation.

# Materials & Methods

*Chemicals and Bacterial strains*

Solid Atrazine (98.8% purity) was purchased from Sigma-Aldrich. P.ADP strain was generously donated by the University of Minnesota BioTechnology Institute. *Escherichia coli* JW0586-1 [Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), lambda-, rph-1, Δ(rhaD-rhaB)568, hsdR514 entE732(del)::kan] with resistance to kanamycin was obtained from the E. coli genetic Stock Center, and used for conjugation experiments.

*Atrazine Detection*

Samples containing atrazine were filtered with 0.40μm hydrophilic Teflon syringe filters. Atrazine was isolated from filtered samples by liquid-liquid extraction with hexane. Hexane solutions containing atrazine were measured for atrazine concentration by gas chromatography – mass spectrometry (GC-MS). The temperature cycle for analysis is outlined in Varian GC/MS/MS detection of atrazine (Peron et al., Varian), and the mass spectrometer was run in SIM mode with peak detection at 200, 202, and 250 amu.

*Culture Conditions*

P.ADP cells were grown in 125mL flasks containing atrazine minimal media. Cultures were maintained at $37^0$C and a shake velocity of 170rpm. Atrazine minimal media was composed of atrazine (217ppm on plates, 37ppm in liquid media), glucose (3.1g/L), sodium citrate (2g/L), 67mL/L phosphate buffer (pH 6.8), 5mL/L R-salt (16.1g

$MgSO_4*7H_2O$, 0.4g $FeSO_4*7H_2O$ and 0.9mL concentrated HCl in 200mL), and 1mL/L Trace Elements (14mg $ZnSO_4*7H_2O$, 5mg $MnCl_2*4H_2O$, 33mg $H_3BO_3$, 26mg $CoCl_2*6H_2O$, 1.9mg $CuCl_2*2H_2O$, 2.5mg $NiCl_2*6H_2O$ and 3.4mg $Na_2MoO_4*2H_2O$ in 1000mL $H_2O$). Atrazine was not added to liquid media until directly prior to inoculation. P.ADP and *E.coli* were also cultured on nonselective Lysogeny Broth (LB) media.

*Mutagenesis procedure*

Liquid cultures were allowed to grow for two days, and then diluted to 1/100,000 in $DiH_2O$ for an average of 170 million cells per dilute culture. Dilutions were gently vortexed to separate cells, and were irradiated with short wave UV light at a distance of 8 cm. For each sample, 50µL or 100 µL of irradiated culture was transferred onto an atrazine plate and allowed to grow for two days at $37^0$C. The same dilute culture was used for each sample in cell survival curves, and the UV lamp was turned off briefly between samples. Time zero samples were taken from dilute culture directly prior to irradiation and percent cell survival was determined from the ratio of colonies formed on plates from samples taken before and after irradiation.

*Mutant enrichment and isolation*

Faster growing mutants were enriched in culture by diluting growing cultures in new media, incubating for two days, and diluting cultures again in new media. Transfer volume was reduced in each enrichment, from 10mL culture in 20mL new media, to 5mL culture in 25mL new media, and finally to 1mL culture in 29mL new media. After three rounds of enrichment for faster growth, individual mutants were isolated by serial

dilution of culture and inoculation on atrazine plates. The largest colony on the plates was then isolated and grown in liquid culture. Growth of the mutant and control strains was measured by optical density at 600nm using a UV-visible spectrophotometer.

*Mating Experiments*

A double conjugation experiment was performed to determine whether genetic mutations occurred in the bacterial chromosome or in the atrazine degrading plasmid. P.ADP (donor) and *E. coli* (recipient) strains were cocultured in LB broth. Atrazine degrading *E.coli* were isolated by inoculating coculture on atrazine plates supplemented with kanamycin (50µg/mL) and transferring growing colonies on new media. Atrazine plates containing nalidixic acid (20µg/mL) were used as a negative control. In addition, wild type P.ADP was cultured in nonselective LB media and replica plated from LB to atrazine plates to test for spontaneous loss of atrazine metabolism. These $Atz^-$ colonies were then isolated and used as the recipient strain for the second conjugation experiment.

In the second conjugation experiment, $Atz^-$ P.ADP and $Atz^+$ *E.coli* were cocultured in nonselective LB media and transferred to atrazine solid media supplemented with nalidixic acid. Colonies growing on atrazine media were isolated on new media, and tested for contamination using atrazine plates supplemented with kanamycin as a negative control. Growth of the rescued strain was then compared to growth of the control and mutant strains by optical density.

# Results

*Atrazine Detection by GC-MS*

The GC-MS readily detected atrazine in solution at 250ppm. No atrazine peaks were detected in 64ppm atrazine samples, or in samples extracted from bacterial culture. Applying an internal standard to samples allowed for atrazine peak detection (Figure 3), however, results were inconclusive due to high variability in peak height. Because of the high uncertainty in the data, atrazine quantification by GC-MS was abandoned in favor of bacterial growth measurements.

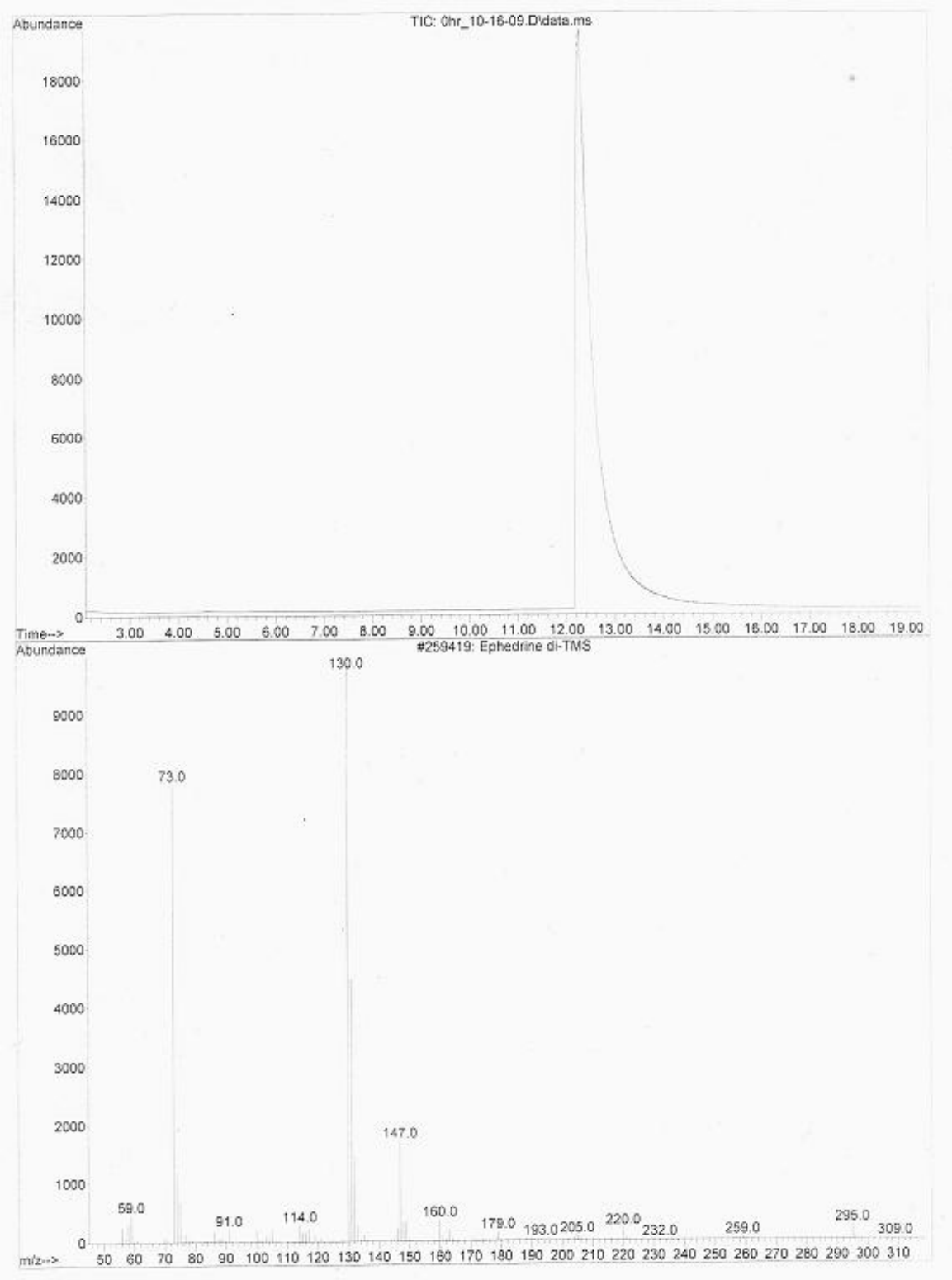


Figure 3 – GC-MS spectra for atrazine – hexane solution extracted from atrazine liquid media inoculated with P.ADP. Isotopic mass to charge ratios (m/z) are consistent with atrazine standard data and literature spectra.

*Growth of P. ADP and E.coli in subculture*

Colonies of P.ADP were visible on solid media after two days of incubation at $37^0$C. Colonies appear off-white on atrazine plates (Figure 4), and flocculent colonies have a pinkish tinge in liquid culture. Colonies on atrazine plates showed distinct clearing zones days after inoculation, and remained viable weeks after the initial colonization. P.ADP grown in the presence of nalidixic acid showed much slower growth, and no growth was evident on plates supplemented with kanamycin. *E.coli* grew readily on LB media and LB supplemented with kanamycin, but did not grow in atrazine media, or on LB plates supplemented with nalidixic acid.


Figure 4 – Wildtype P.ADP colonization on an atrazine plate

*UV mutagenesis results*

Results from various dilution series indicated that a hundred thousand fold dilution and 50µL injection volume gave the best colony separation for cell counting. Cell survival was 57% after only five seconds of irradiation of short wave ultraviolet light (Figure 5), and no colony growth was visible after half a minute of exposure to UV light (Figure 6). A five second exposure time was used for mutant screening.

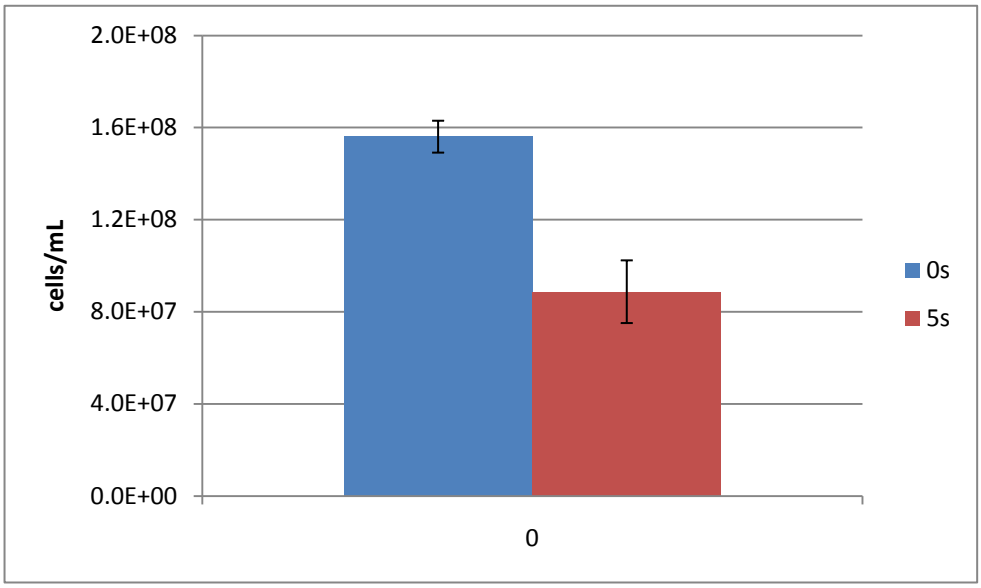


Figure 5 – Cell survival of P.ADP after five seconds of exposure to UV irradiation

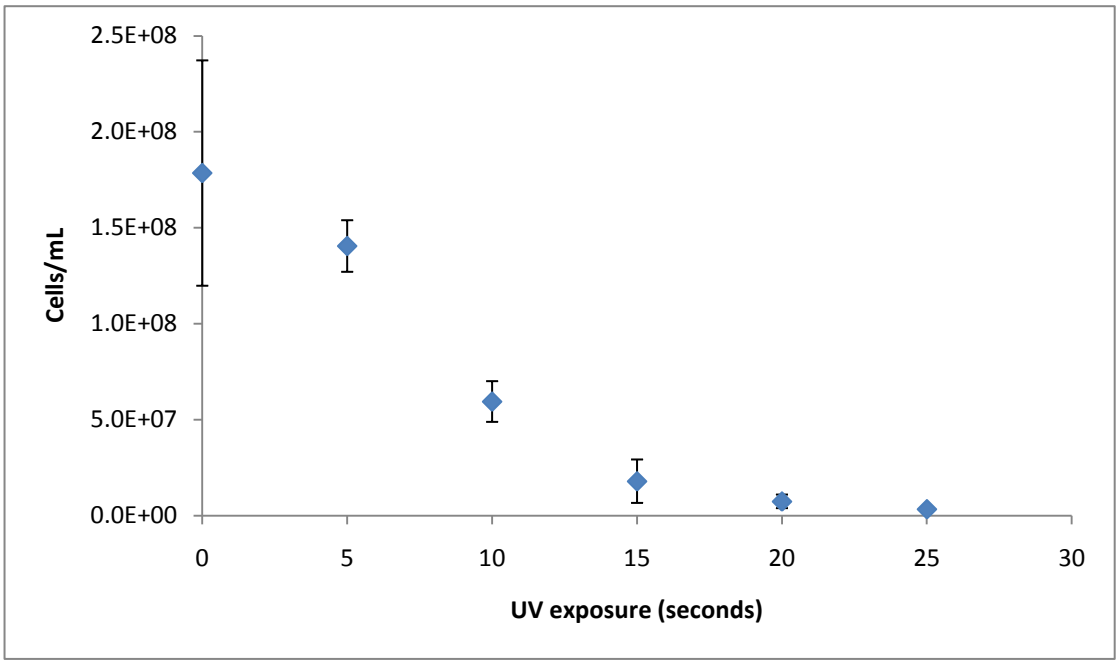


Figure 6 – Cell survival curve of P.ADP after exposure to short wave UV light

Three colonies with the largest growth on plates were selected for analysis. These cells, labeled M1, M2 and M3, were isolated from three separate mutation and enrichment experiments. Cultures of M1 and M2 grew in liquid culture at similar rates to the control strain. Bacteria from colony M3, however, consistently grew at a faster rate than the control strain in atrazine liquid media. A faster growth rate was evident during the early stages of growth, and the mutant demonstrated a slightly higher rate of growth throughout the exponential growth period (Figure 7). The mutant also reached a higher density than the control strain, and maintained higher optical density seventy two hours after exponential growth had ended (Figure 8).

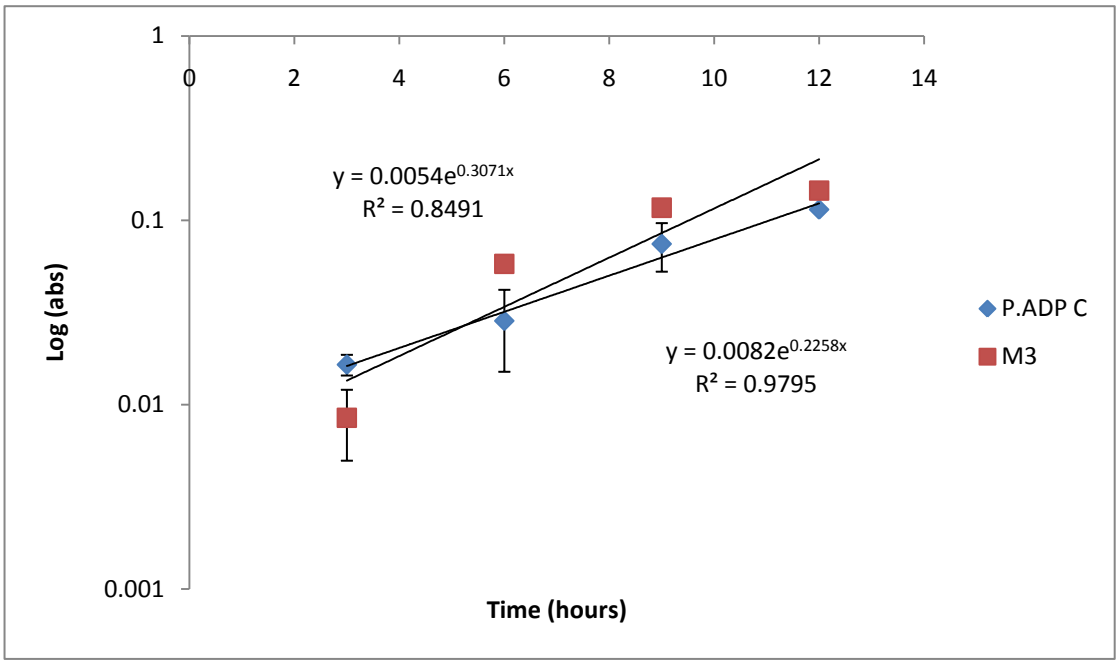


Figure 7 –Exponential growth periods of the P.ADP control strain (P.ADP C) and mutant strain (M3) in atrazine minimal media.

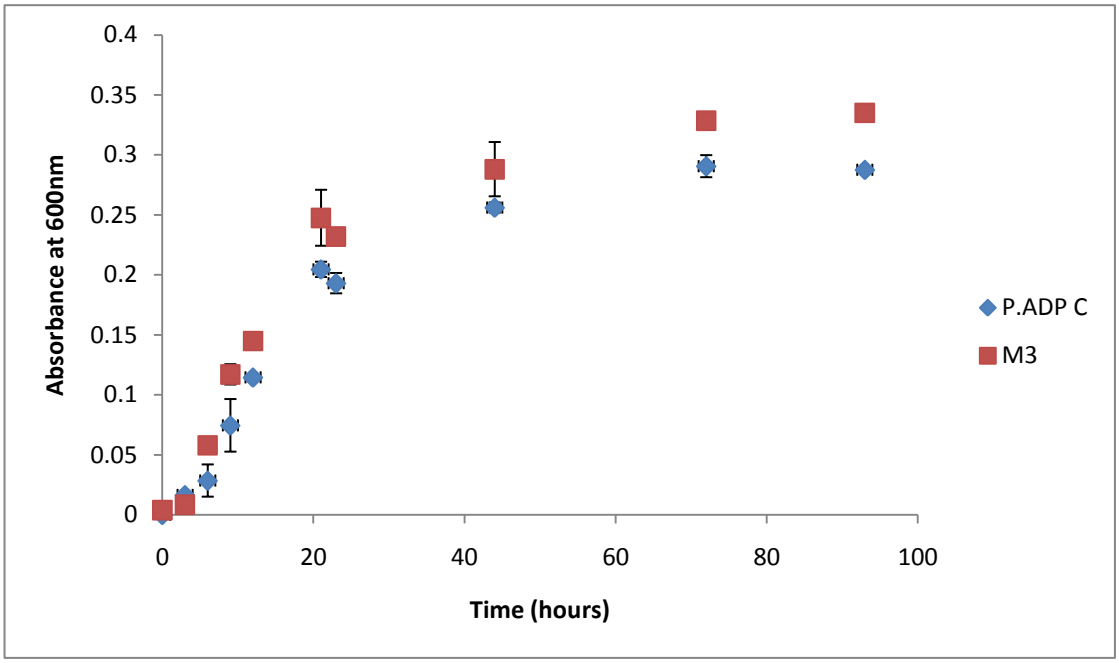


Figure 8 - Growth curves for P.ADP control strain (P.ADP C) and the P.ADP mutant strain (M3) in atrazine minimal media.

In enriched, atrazine free media, M3 cells grew at a similar, or even slower rate than the control strain (Figure 9). Growth rate data are, however, difficult to interpret because of the higher optical density of the mutant cultures compared to the controls. Higher cell abundance of the mutant in the exponential data may be due to slightly higher transfer volume in the M3 culture during inoculation, and repeating the experiment resulted in the control strain growing faster in the exponential period than the mutant strain. In addition, though M3 maintained higher optical density than the control fifteen hours after exponential growth had ended, this experiment was not repeated, or carried out for a longer time period. Though optical density data suggest there is little difference in growth between M3 and the control in atrazine free media, additional data are necessary before drawing such a conclusion.

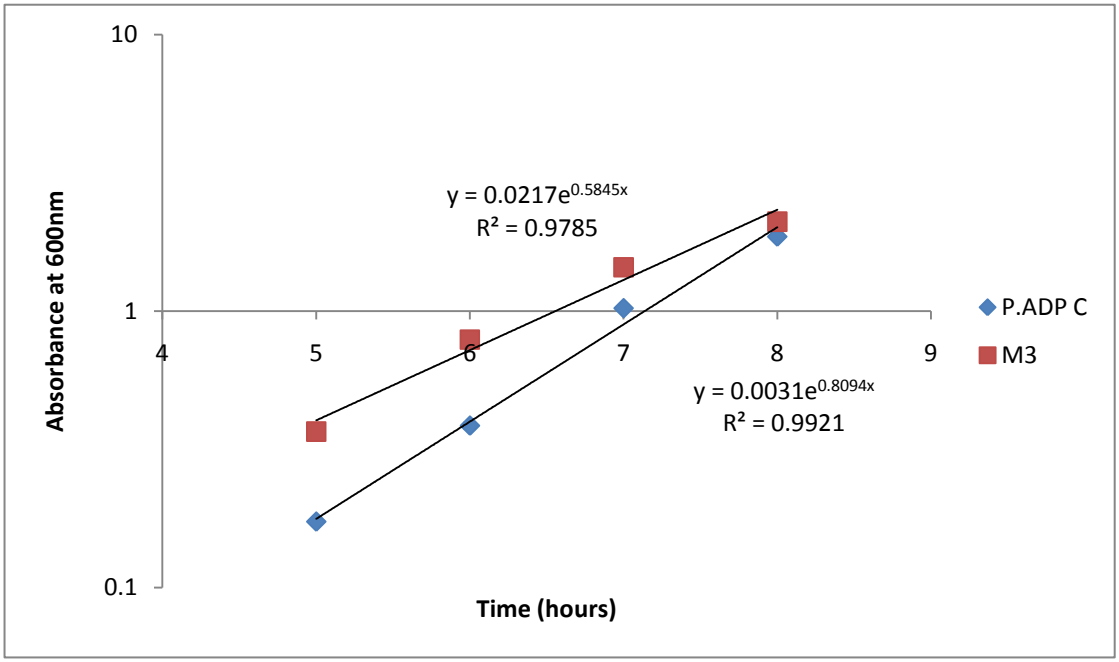


Figure 9 – Exponential growth periods of the P.ADP control strain (P.ADP C) and mutant strain (M3) in atrazine free LB media.

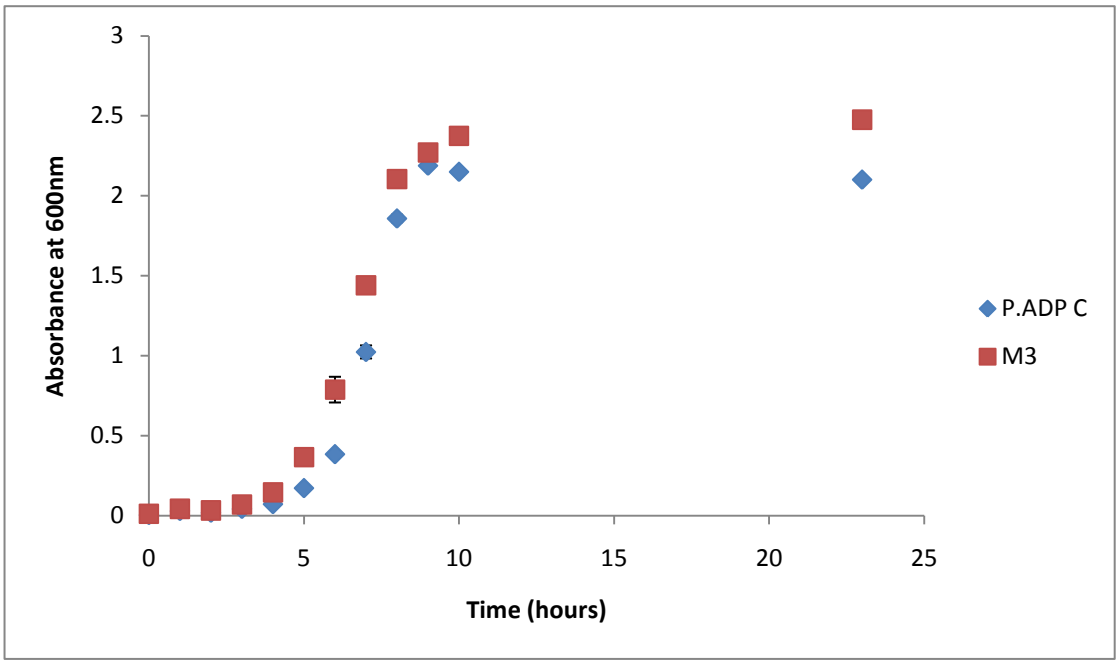


Figure 10 - Growth curves for P.ADP control strain (P.ADP C) and the P.ADP mutant strain (M3) in atrazine free LB media.

*Mating Experiments*

Growth of P.ADP and *E.coli* Kan$^r$ in the presence of either kanamycin or nalidixic acid showed that the two chemicals were species selective at our concentrations. *E.coli* cocultured with both P.ADP and M3 showed small but visible growth when transferred to atrazine plates containing kanamycin. These colonies did not grow on atrazine plates supplemented with nalidixic acid, indicating that colonies were atrazine degrading *E.coli*, and not P.ADP that had developed a resistance to kanamycin.

P.ADP colonies on LB plates showed the same pattern of colonization when replica plated to new LB, but showed regions of no growth when transferred to atrazine plates (Figure 11). Colonies that corresponded to no growth on atrazine plates were isolated, and additional replica plating confirmed that these colonies had lost the ability to metabolize atrazine.

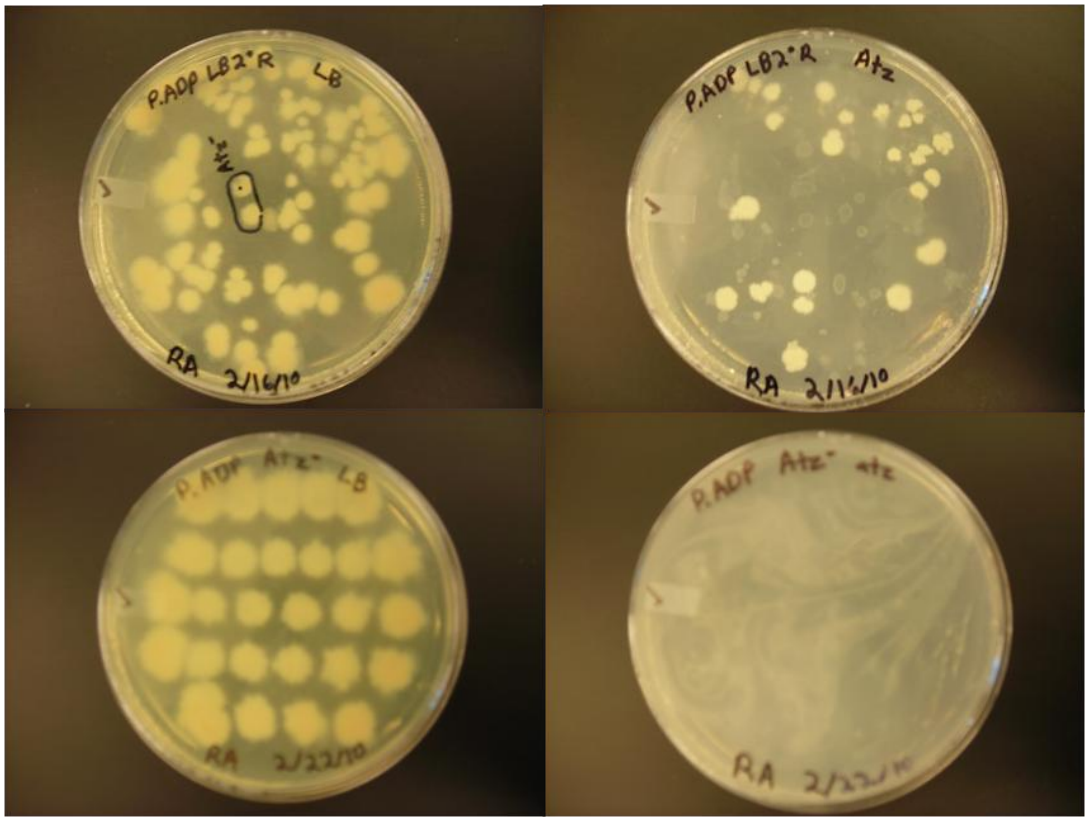


Figure 11 – Spontaneous loss of atrazine metabolism. P.ADP grown on LB (top left) replica plated onto atrazine plates show areas of no growth (top right). Suspected mutant colonies were isolated and grown on LB (bottom left) and replica plating onto atrazine plates resulted in no growth (bottom right).

P.ADP Atz$^-$ was cocultured with either *E.coli* Atz$^+$ or *E.coli* Atz$^{M+}$ in LB broth, and both cocultures showed small but visible growth when transferred to atrazine plates containing nalidixic acid. Isolated colonies did not grow on atrazine plates supplemented with kanamycin, suggesting that colonies were P.ADP with rescued atrazine degrading function, and not *E.coli* Atz$^+$. The presence of nalidixic acid strongly inhibited the growth of P.ADP, though colony formation was visible after a week of incubation. Colony growth of the second conjugate never reached a density where the growth rate could be measured, even after transfer to nalidixic acid free atrazine media.

# Discussion

In this experiment we have isolated a novel growth mutant of P.ADP after three independent rounds of random mutagenesis. The isolated mutant strain grows both faster and to a higher total abundance than the control strain in minimal media with atrazine as the sole nitrogen source. Atrazine concentration in culture could not be measured directly, as a low limit of detection and high uncertainty in peak height made results unreliable. However, in media with atrazine as the sole nitrogen source, atrazine levels and cellular growth are expected to have a negative correlation, and, in the present study, growth data could be used as a surrogate for atrazine concentration.

Though we could not determine atrazine concentration directly, growth of the mutant and control strains in LB suggests that the mutant's higher growth rate is dependent on atrazine concentration. The mutation in strain M3 may promote the activity of atrazine catabolic genes on the plasmid, or the mutation may facilitate uptake of atrazine into the cell. However, further study of M3 in atrazine free media is needed to confirm that the higher growth rate of M3 is dependent on atrazine concentration.

Previous studies found that P.ADP grown in enriched media can spontaneously lose the ability to degrade atrazine. We demonstrate here that P.ADP frequently loses atrazine degrading function in enriched media. $Atz^-$ mutants may originate from nonfunctioning or absent genes coding for key enzymes in the atrazine degradation pathway, and some have reported a lack of the atzA, atzB and atzC genes in $Atz^-$ mutants (de Souza et al., 1998). We believe the lack of the atzABC genes reported in $Atz^-$ mutants is due to a loss of the entire atrazine degrading plasmid in the bacteria, from an uncoupling of plasmid

replication and cellular division in atrazine free, enriched media. The instability of the atrazine degrading phenotype has important consequences in laboratory scale procedures, but may play a lesser role in field studies and applications, where there is high competition for limited resources.

Though unnecessary for the current project, a method for quantifying atrazine concentration in culture is important for further study of the mutant strain. Atrazine quantification is important in determining enzyme kinetics, substrate affinity, and enzyme specificity for atrazine. A reliable procedure for determining atrazine concentration is especially important in studying atrazine degradation in complex media, where multiple nitrogen sources are present. The spectrophotometric method of atrazine quantification used in González et al. (2003) may serve as a rough estimate of atrazine concentration when other methods, like GC-MS, are unavailable or unreliable.

In addition to biotic factors such as gene expression and enzyme structure, abiotic factors play a significant role in microorganisms' ability to breakdown persistent organic compounds. The primary abiotic limitation to remediation is the bioavailability of the compound of interest (Dua et al., 2002; Pieper & Reineke, 2002). Bioavailability is limited by the low solubility of many xenobiotics. The sorption potential of these compounds can be improved by increasing their solubility in water from introducing surfactants, and some microorganisms can secrete biosurfactants independently to increase uptake potential. Though surfactants can increase the bioavailability of xenobiotics for catabolism, they can also limit the adhesion potential of cells and therefore limit the colonizing ability of bacteria. Thus, the overall benefit of surfactants for remediation is uncertain. Other limiting factors for bioremediation are resilience to

toxic pollutants as well as interference from nontarget pollutants. Microorganisms must be equipped with some method of toxicity resistance to a variety of pollutants if they are to function for *in situ* applications. The availability of more desirable nitrogen sources is perhaps one of the most important factors in the use of P.ADP in atrazine bioremediation. González et al. (2003) found that the presence of other nitrogen sources had a significant negative impact on Pseudomonas' ability to degrade atrazine. Preferable catabolism of alternative nitrogen sources may have a significant impact in field applications; however, inhibiting alternative nitrogen metabolism in the strain may help avoid this issue (González et al., 2003).

The recent evolution of atrazine metabolism suggests that genetic manipulation may significantly improve catabolic efficiency. Random mutation by DNA shuffling has been reported to improve atrazine degradation 80-fold in P.ADP (Patten et al., 1997), and a study by Scott et al. reported a 20-fold improvement in atrazine chlorohydrolase $k_{cat}/K_m$ after combinatorial randomization of the atzA gene (2009). However, a separate study by Seffernick and Wackett found only a 1.4-fold increase in atzA gene activity after DNA shuffling (Seffernick & Wackett, 2001). If randomization experiments are performed on other genes in the plasmid, we can asses which genes in the pathway are most amendable to genetic manipulation. Irrational mutation, mutagenesis, selection of desirable mutants, purification and additional mutation, mimic the power of natural evolution to select for organisms with novel features, though at a much higher rate. This selection could be designed for any parameter of interest, be it atrazine degradation, general growth, toxin tolerance, enzyme production, biosurfactants production, or sorption. In addition, studying the genomic differences between mutated strains and controls will help in

understanding the functional significance of the amino acid sequence and show potential sites for rational mutagenesis.

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