ENGINEERING pH TOLERANT MUTANTS OF A CYANIDE DIHYDRATASE OF *BACILLUS PUMILUS* C1 AND IDENTIFYING CONSTRAINTS ON SUBSTRATE SPECIFICITY IN NITRILASES

A Thesis

by

LAN WANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2008

Major Subject: Microbiology

ENGINEERING pH TOLERANT MUTANTS OF A CYANIDE DIHYDRATASE OF *BACILLUS PUMILUS* C1 AND IDENTIFYING CONSTRAINTS ON SUBSTRATE SPECIFICITY IN NITRILASES

A Thesis

by

LAN WANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Michael J. Benedik
Committee Members,	Susan S. Golden
	James R. Wild
Head of Department,	Vincent M. Cassone

May 2008

Major Subject: Microbiology

ABSTRACT

Engineering pH Tolerant Mutants of a Cyanide Dihydratase of *Bacillus pumilus* C1 and Identifying Constraints on Substrate Specificity in Nitrilases. (May 2008)

Lan Wang, B.S., Beijing Normal University;

M.S., Institute of Medicinal Biotechnology, Peking Union Medical College

Chair of Advisory Committee: Dr. Michael J. Benedik

This study generated two cyanide dihydratase (CynD) mutants of *Bacillus pumilus* C1 with improved activity at higher pH by random mutagenesis. The purpose of this study was to create enzyme variants better suited to degrade cyanide under the harsh conditions of industrial applications. We employed error-prone PCR to construct a library of CynD mutants. A high throughput screening system was developed to screen the library for improved activity. Two mutants were identified that could degrade cyanide at pH10 whereas the wild-type enzyme was inactive at pH9 or higher. The mutants each had three amino acid substitutions compared to the wild-type enzyme. The mutants were also more stable than the wild-type enzyme at 42^{o}C. E327G was identified as one of the key amino acids that are responsible for the improved activity.

The goal of the second project was to convert substrate specificity of the *Bacillus* sp. OxB-1 nitrilase to that of a cyanidase by mutagenesis or construction of hybrid genes. The OxB-1 nitrilase of *Bacillus* sp. shows a high level of identity with the cyanide dihydratases from *B. pumilus* C1 and *P. stutzeri* AK61 but utilizes different substrate.

This provides a valuable resource to study the substrate specificity determinants of cyanide degrading enzymes. One deletion mutant and four hybrid proteins were constructed based on the alignment information. The constructed proteins were all unable to degrade cyanide.

DEDICATION

This thesis is dedicated to my family.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Michael J. Benedik, for providing me with the opportunity to work in his laboratory and for guiding and supporting me throughout my research. He was always there to talk about ideas and helped me to solve technique problems; he enjoys working in the lab so much which encouraged me through the tough time when the experiments did not work; and he helped me edit the manuscripts of my proposal and thesis with great patience.

I also would like to thank my committee members, Dr. Susan S. Golden and Dr. James R. Wild, for their guidance, comments and questions on my research. All the processes developed my scientific and critical thinking.

Thanks go to many labmates and friends for their help in the passing years. They made my time at Texas A&M University a great experience.

Finally, thanks to my mother, father, and sister, for their always being there for me.

NOMENCLATURE

epPCR	error-prone PCR
CynD	cyanide dihydratase
CHT	cyanide hydratase
$CynD_{pum}$	cyanide dihydratase from *Bacillus pumilus* C1
$CynD_{stut}$	cyanide dihydratase from *P. stutzeri* AK61

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Cyanide is a very important chemical in a variety of industrial processes. The resultant cyanide-containing waste must be detoxified before its release to the environment. Compared with conventional methods to detoxify cyanide, microbial treatment is an inexpensive and environmentally friendly method. Cyanide-degrading enzymes have been found in a number of bacteria (cyanide dihydratases), fungi (cyanide hydratases) and plants.

Cyanide-containing wastes differ in pH and solute content from the different uses of cyanide in industrial processes. Effective candidates for biological treatment of cyanide should tolerate adverse conditions in addition to the mere ability to degrade cyanide compounds.

Error-prone PCR and DNA shuffling are effective directed evolution methods to generate mutant libraries to screen for desired properties. Since the catalytic mechanism and protein structural information of cyanide dihydratases have not been determined as of yet, more information about these will be gained from the study of directed evolution and the substrate specificity of this enzyme.

Cyanide and detoxification of cyanide containing waste

Hydrogen cyanide (H-C≡N) is the simplest of nitriles (R-C≡N). HCN is a weak

---

This thesis follows the style of Journal of Bacteriology.

acid and comes from both natural and manufactured sources. Cyanide is completely soluble in water at 25^{o}C but it volatilizes easily with a boiling point of 25.7^{o}C (19). Cyanide exists in the environment in several forms, such as hydrogen cyanide, simple inorganic salts (NaCN and KCN), and complex metal cyanides. Free cyanide (HCN or CN^-) is an extremely potent metabolic poison and metal-cyanide complexes or nitriles vary in toxicity according to the degree of cyanide liberation by these compounds (15). Recent research in the United States and Europe suggests that cyanide is an important cause of smoke-inhalation morbidity and mortality and cyanide continues to be an ideal chemical weapon (4). The lethal or toxic effect of cyanide is due to its affinity for cytochrome oxidase which will inhibit the electron transport chain and cause cessation of aerobic cell metabolism (44). Moreover, chronic ingestion of cyanide at sublethal doses may cause certain neuropathies and paralysis (40).

Despite its toxicity, cyanide is in substantial demand in a variety of industrial processes. The economic importance of cyanide is its ability to form tight complexes with a variety of metals to make them soluble. Cyanide compounds used in industry include hydrogen cyanide, cyanide salts containing sodium or potassium, gold and silver cyanide, etc. In 2004, the United States produced and used an estimated 1.838 billion pounds of cyanide (34). The mining industry relies heavily on sodium cyanide to extract gold, and cyanide leaching technology accounts for 90% of worldwide gold production. Other industries such as electroplating, plastic manufacturing, photography, photoengraving, and pesticide production either use or generate cyanide compounds during processing.

The large volumes of cyanide-containing waste generated during industrial production and processing are a real threat to nearby ecosystems and cyanide-related accidents in the mining industry have caused human casualty and environmental damage worldwide. Therefore, cyanide waste must be detoxified before release to the environment. Most cyanide remediation processes operate on the principle of converting cyanide into one or more less toxic compounds. There are several conventional physical and chemical treatments of cyanide waste in commercial applications. The most wildly used detoxification method is chemical oxidation of cyanide including the INCO process and the hydrogen peroxide process (43). The chemistry of these two processes is summarized as following:

INCO Process: $CN^- + SO_2 + O_2 + H_2O \rightarrow OCN^- + SO4^{2-} + 2H^+$

Hydrogen peroxide process: $CN^- + H_2O_2 \xrightarrow{Cu^{2+}\ catalyst} OCN^- + H_2O$

For the INCO process, pH needs to be adjusted by the addition of lime. Both processes are ineffective against cyanide complexes with iron, gold and silver (3). Costs are still a concern when treating higher concentrations of cyanide with the above processes. Other physical processes to remediate cyanide waste include granular activated carbon, cyanide recovery, and natural attenuation. These methods are very useful to lower the very high cyanide concentrations present in tailings slurries, but they require secondary treatment to eventually remove all cyanide (30).

Microbial treatment or bioremediation may provide a potentially inexpensive, environmentally friendly alternative to conventional methods, since none of the physical

or chemical treatments currently employed fully satisfies the detoxification of cyanide-containing waste. The main advantages of biological treatment are its ease and low cost of operation. But the biological treatments are susceptible to environmental conditions, for example pH, temperature, and the presence of additional pollutants. Therefore, besides the ability to degrade cyanide compounds, one should also consider whether enzymes are tolerant of the additional stresses when seeking effective candidates for bioremediation of cyanide waste.

Cyanide-degrading enzymes have been found in a number of bacteria, fungi and plants. Microorganisms can degrade cyanide by hydrolysis, oxidation, reduction or substitution (13). Most of these enzymes require additional substrates or cofactors to degrade cyanide. Thus, they are unlikely to be considered as means of bioremediation of cyanide waste.

Nitrilase and substrate specificity

Enzymes of the nitrilase superfamily hydrolyze a variety of nonpeptide carbon-nitrogen bonds utilizing a characteristic Glu-Lys-Cys catalytic triad; these enzymes are classified into 13 branches (8). Nitrilases (E.C.3.5.5.1) comprise the only branch of the nitrilase superfamily that can convert nitriles (RCN) to the corresponding acids and ammonia. Nitrilases use a broad range of nitriles as substrates with important applications in biotransformation to produce fine chemicals for the pharmaceutical industry (6). This nitrilase branch includes cyanide dihydratase (cyanidase) and cyanide hydratase. Cyanide dihydratase and cyanide hydratase use cyanide (HCN), which is the

simplest nitrile, as substrate while showing little activity towards other nitriles. These cyanide-degrading enzymes provide great potential for the bioremediation of cyanide waste. Nitrilases and cyanide dihydratases produce acid and ammonia whereas cyanide hydratases produce formamide as their product:

$$RCN \xrightarrow[\text{Nitrilase}]{} RCOOH$$

$$HCONH2 \xleftarrow[\text{Cyanide hydratase}]{} HCN \xrightarrow[\text{Cyanide dihydratase}]{} HCOOH + NH_2$$

Nitriles serve as useful starting materials for synthetic organic chemicals, pharmaceuticals and agrochemicals (6). Nitriles can be synthesized through different methods and the enzymatic dehydration of aldoximes is one of the most useful methods because many chemical methods require harsh conditions. The nitrilase gene from *Bacillus* sp. strain OxB-1 is linked to the gene for phenylacetaldoxime dehydratase which catalyzes the degradation of aldoxime to nitrile (20). The nitrilase of OxB-1 participates in aldoxime metabolism and hydrolyzes phenylacetonitrile to phenylacetic acid (20). It will become of interest to my work because of its remarkable similarity to cyanide dihydratases.

Compared with other cyanide-degrading enzymes, cyanide dihydratases and hydratases are good candidates for use in the bioremediation of cyanide waste because they do not require additional cofactors or substrates during hydrolysis (18). All cyanide hydratases found to date are fungal in origin including *Fusarium lateritium*, *Gloeocercospora sorghi*, and *Leptosphaeria maculans*. Since the product of cyanide hydratase is formamide, this group of enzymes catalyses a monohydrolysis of the

substrate; therefore the reaction is a nitrile hydratase reaction rather than a nitrilase reaction. In contract, cyanide dihydratases are true nitrilases and convert cyanide to the corresponding carboxylic acid and ammonia. Cyanide dihydratases have been found in only a few bacteria including *Alcaligenes xylosoxidans* subsp. *Denitrificans* DF3 (16), *Bacillus pumilus* C1 (22), and *P. stutzeri* AK61 (39). The cyanide-degrading strain *Bacillus pumilus* C1 was isolated from a soil sample near a cyanide-containing waste water dam in South Africa (21). This enzyme cannot hydrolyze cyanate, thiocyanate, azide or acetonitrile, showing specificity for cyanide (22).

A comparative study of cyanide-degrading nitrilases was performed in our lab with the respect to their pH stability, thermostability, metal tolerance, and kinetic constants (18). The pH activity profile shows that the optimal pH of $CynD_{pum}$, $CynD_{stut}$, and CHT of *G. sorghi* are all in the range of pH7-8, but CHT is more tolerant of higher pH. CHT retains about 80% relative activity at pH8.4, whereas the CynD enzymes rapidly lose activity above this pH. The activities of all three enzymes decrease monotonically as pH decreased below 7. CHT is less sensitive to Hg^{2+} and more sensitive to Pb^{2+} than the CynD enzymes. Both CynD enzymes have maximal activity between 37°C and 42°C, while CHT has maximal activity between 42°C to 55°C. The thermostability study shows that $CynD_{pum}$ is most stable, losing no activity when incubated at 23°C and maintaining over 50% activity when incubated at 42°C for over 24 hours and with intermediate thermostability at 37°C. $CynD_{stut}$ quickly loses activity even at 23°C and 37°C. CHT is the least themostable of the three enzymes and lose about 70% activity at 37°C. All enzymes are inactivated completely when incubated at 55°C for 30 minutes. CynD

enzymes have similar K_m (6-7 mM) and V_{max} (0.1 mmol $min^{-1}mg^{-1}$) while CHT has much higher K_m and V_{max} from the data obtained in this study and previously published data.

These properties of CynD and CHT suggest the existing cyanide-degrading nitrilases all have their limitations for use as agents in bioremediation under conditions needed for industrial applications. This points out the benefit of engineering the cyanide-degrading enzymes for improved properties relevant to industrial cyanide remediation.

A comparative analysis of their amino acid sequences shows that cyanide hydratases are very closely related to each other with an identity of 74.1-88.7% (http://www.ebi.ac.uk/clustalw/). The two cyanide dihydratases have an identity of 75.8% and the homology between cyanide hydratases and dihydratases is 46.8%. The nitrilase group shows much less conservation within the group and the general degree of identity is much less than 50% (27). One exception is notable, the nitrilase of OxB-1 shows a high level of identity (～70%) to the cyanide dihydratases from *B. pumilus* C1 and P. *stutzeri* AK61. These enzymes are similar at the amino acid level but the functional difference between them provides a valuable resource to identify substrate-specificity determinants.

Quaternary structure and modeling of nitrilases

Atomic resolution structures of eleven members of the nitrilase superfamily have been determined. All these enzymes are distantly homologous, but they share a typical

αββα fold with the conserved glutamate, lysine and cysteine catalytic site. These enzymes exist as dimers, tetramers, hexamers or octamers.

Until now, crystal structures of the microbial nitrilases, including cyanide dihydratase and cyanide hydratase, still remain elusive, although many studies have probed the structure of these enzymes. Previous studies demonstrate that members of the nitrilase superfamily should have homologous structure despite their difference in sequences and substrates (28). Using low-resolution electron microscopy combined with homology modeling of the available nitrilases structures, models of the structure of the microbial nitrilases have been created. The microbial nitrilases usually form active homo-oligomeric spirals with different numbers of subunits (32). The oligomers are comprised of dimers with a subunit size between 30 to 45kDa (27). Evidence suggested that oligomer formation is essential for the activity (32). Multiple alignments showed that the microbial nitrilases differ from the nonspriral-forming nitrilases of determined structures by two insertions (12-14 amino acids) and a C-terminal extension of up to 35 amino acids (32). These residues were positioned at the interacting region in the homology model and are believed to contribute to the formation and/or stabilization of the spiral quaternary structure (32).

The quaternary structure of cyanide dihydratases from *B. pumilus* C1 and *P. stutzeri* AK61 and cyanide hydratases from *G. sorghi* was determined by three-dimensional reconstruction. The two cyanide dihydratases both comprise two-fold symmetric spiral structures under conditions of optimal activity (pH7-8) (17, 31). Cyanide dihydratase from *B. pumilus* C1 is an 18 subunit-spiral structure composed of dimers with the

37kDa-subunit (17). Cyanide dihydratase from *P. stutzeri* AK61 form a 14-subunit spiral and the subunit size is 38kDa (Fig. 1) (31). The quaternary structure of cyanide dihydratase from *B. pumilus* C1 is similar to that of *P. stutzeri* AK61 as shown in Fig. 1. Cyanide hydratases from *G. sorghi* exist as long regular helices with 40kDa-subunit (42, 36).

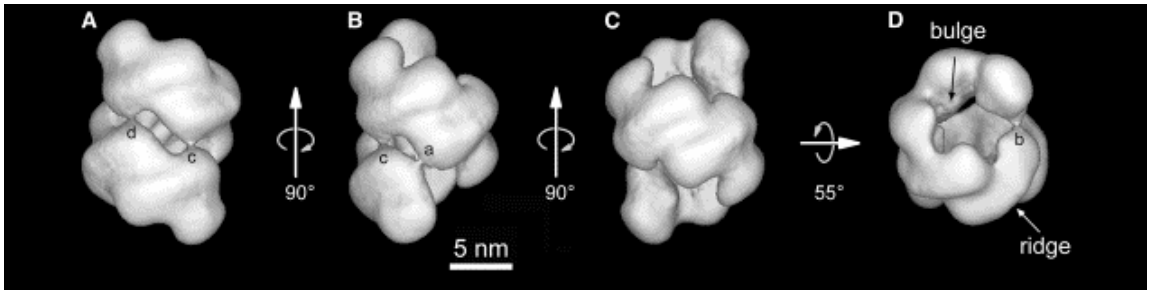

FIG. 1. The three-dimensional map of the negatively stained cyanide dihydratase from *P. stutzeri* AK61. The global 2-fold axis is apparent in views (A) and (C). "a, b, c, d" indicate the interacting regions between the subunits occur across the groove. The quaternary structure of cyanide dihydratase from *B.pumilus* C1 is similar to that of *P.stutzeri* AK61. (adapted from reference 31)

Two interesting findings about cyanide dihydratase from *B. pumilus* C1 should be noted. The structure of this enzyme changes from an octadecamer at pH8 to a long helical fiber at pH5.4 (17). It was believed that no major structure rearrangement occurred other than increasing the length of the spirial, and the histidine residues in its C-terminus may contribute to this change in oligomeric state (17). A highly homologous enzyme from *B. pumilus* A3 lacks these histidine residues and does not show this transition. An increase in this enzyme's activity at this pH5.4 inflection point was also observed, unlike other cyanide-degrading enzymes (18). This activity change was due to

the number of the available subunits which would change in this structural transition because the terminal dimers are predicted to be inactive.

Another finding is that *B. pumilus* C1 enzyme was more robust to C-terminal truncations while very small changes affected the activity of cyanide dihydratase from *P. stutzeri* AK61 (32). *B. pumilus* C1 enzyme still functioned until its C-terminal residue was truncated back to 293. *B. pumilus* C1 enzyme functioned with the C-terminus of *P. stutzeri* AK61 enzyme, but *P. stutzeri* AK61 enzyme cannot function with the C-terminus of *B. pumilus* C1 enzyme. From the above effects of the C-terminus and the studies of the other microbial nitrilases, it is suggested that the C-terminal region may be important but not always essential for quaternary structure and stability of the enzyme.

Overall, the information from the determined nitrilase structures can be applied in the modeling the structures of the microbial nitrilases. However, the lack of a microbial nitrilase crystal structure obstructs better understanding of the substrate specificity, mechanism of catalysis, and the optimization of these enzymes.

Directed evolution: strategies for randomization and library construction

Directed evolution resembles Darwinian evolution in a test tube. The goal is to generate a protein with desired properties by screening or selecting from a large constructed library of protein mutants (14, 5). Despite our increased knowledge of protein structure and function in recent years, we still cannot predict many aspects of an enzyme mechanism. Compared with rational design methods, a significant advantage of directed evolution is that no structural or mechanistic information is required, and it can

uncover unexpected beneficial mutations (9).

The goal of most directed evolution strategies is achieved by making subtle changes to the wild- type enzymes (7). This outcome is based on the fact that most enzymes have weak promiscuous activities which are quickly improved by only a few mutations (1). Untill now the most interesting targets for directed enzyme evolution have been catalytic activity, substrate specificity, thermal and oxidative stability, enantio-selectivity or enantio-specificity, pH range and tolerance to solvent.

Directed evolution combines two essential components: methods for library construction and strategies for screening or selection for improved variants. A successful screening design is one that can be performed easily with high-throughput technologies and that reflects exactly the stress of an industry application (35).

There are a number of different methods that have been developed to construct a library of variant genes. These methods may be broadly divided into three categories. The first category includes techniques that create genetic diversity by introducing point mutations, deletions and/or insertions at random positions throughout the gene encoding an enzyme. These methods include physical and chemical mutagens, mutator strains, insertion and deletion mutagenesis and different forms of error-prone PCR (epPCR).

Mutating microbes with UV irradiation or alkylating agents has been used for many years. The mutations are created because the damaged DNA is incorrectly replicated or repaired. This idea is also applied in mutator strains. Microorganisms use several different repair pathways to ensure the high fidelity of DNA replication; mutator strains have defects in one or several DNA repair pathways leading to a higher mutation rate.

The *E. coli* XL1-Red strain, commercially available from Stratagene, is an engineered strain deficient in three repair pathways (*mutS*, *mutD* and *mutT*) that has a 5000-fold higher mutation rate than wild-type stains (38). A significant advantage of the above mutagenesis methods is that *in vivo* mutagenesis greatly simplifies the steps in library construction. However, mutagenesis generated by these methods is indiscriminate and leads to many unwanted side-effects such as altered plasmid copy number or gene expression levels. With a high mutation rate of the chromosomal DNA of the host cell itself, the cell may grow much slower and the process of mutagenesis using mutator strains can be quite slow because the level of mutagenesis is controlled by the number of generations in the strain.

The epPCR method is one of the most popular approaches for diversity generation. The principle of this technique is that *Taq* polymerase lacks proofreading activity (a low 5'-3' exonuclease activity) and cannot remove a mismatched base. There are a number of ways to increase the mutation rate in epPCR by modifying PCR conditions, such as addition of Mn^{2+}, increasing the concentration of Mg^{2+}, using unbalanced concentrations of dNTPs, using dNTP analogs, or increasing the number of amplification cycles (11). If higher rates of mutations are needed, a combination of some or all of these methods may be employed. Cirino et al. (11) describe the details to generate an error rate of ~1nt/kb by the addition of Mn^{2+} and an over-concentration of dGTP and dTTP in amplification, and Zaccolo et al. (45) reported that using dNTP analogs in the reaction increases the mutation rate to up to one in five bases. There are also commercial kits available to perform epPCR. The Clontech system (Diversify PCR Random Mutagenesis Kit) uses

Taq polymerase and mutation rates are modified by changing the concentration of Mn^{2+} and dGTP. The Stratagene system (GeneMorph II Random Mutagenesis Kit) uses a highly error-prone polymerase and the mutation frequency is controlled by the concentration of target DNA template and the number of amplification cycles.

Most studies of directed evolution of enzymes begin with epPCR because it is easy to generate mutations by modifying PCR conditions and mutagenesis can be restricted to a region of interest. However, the epPCR method has certain limitations. First, the primary limitation of library size depends on the ligation efficiency of the PCR product into the vector; when large libraries are sought this step can require considerable effort to optimize (33). Second, *Taq* polymerase favors A/T substitutions which results in some types of errors more than others and a bias in the composition of the library (11). A less biased library can be constructed by combining libraries constructed using *Taq* polymerase with those constructed using the Stratagene GeneMorph II kit, which has no base bias, or by employing a newly developed method—Sequence Saturation Mutagenesis (SeSaM). This approach controls the mutation incorporation through a universal base, deoxyinosine (41). In this method, the PCR products are cleaved by iodine to generate random sized DNA fragments tailed by universal bases. The tailed DNA fragments are extended full length by PCR and the universal base is replaced by a standard base.

The third limitation of epPCR comes from the nature of PCR process. PCR is an exponential amplification process and any mutation generated early in the amplification process will be over-represented in the final library. The problem can be solved to some

extent by combining several separate epPCRs to construct a final library or by reducing the number of amplification cycles (25). Different forms of shuffling (discussed below) and random insertion/deletion mutagenesis (24) could also be considered to eliminate the above biases to some extent.

The second category of techniques for library construction is oligonucleotide-based mutagenesis. These techniques are based on the incorporation of a synthetic DNA into the full-length coding DNA sequences and provide a powerful approach to randomly mutate the coding gene at specific chosen positions and regions. The target regions are either interesting positions identified by screening of epPCR libraries or are important domains of the enzyme determined through structural or bioinformatics analysis. The value of this method is that any form of randomization that can be achieved in a synthetic DNA segment can be incorporated into the full-length gene (25). The synthesis of oligonucleotides is well established and most of them can be ordered directly from the suppliers. Therefore, the rate, identity and position of mutations can be completely controlled by control over the chemistry of DNA synthesis (38).

Many methods based on conventional site-directed mutatgenesis can be used to incorporate the synthetic DNA sequence into the full-length gene. The most popular approaches are strand overlap extension and megaprimer-based protocols which are simple to perform (23). Mutagenic plasmid amplification (MPA) (such as the Stratagene QuickChange system) and related methods are also widely used. However, the length of ologonucleotides that can be reliably synthesized limits the size of the region to be randomized. This problem can be solved by performing multiple rounds of muatagenesis

or more complex methods such as gene synthesis. Mutations near the 5'-ends of the primers are more efficiently incorporated than those modified near 3'-ends which leads to a biased library. To reduce this kind of bias, the careful design of primers is needed to provide a reasonable length of fully annealing sequence at the 3'-end (38).

The third category of techniques to construct diversity libraries is the use of recombination methods applied to gene pools generated by the previous methods or derived from nature. The best mutants discovered by directed evolution almost always contain multiple mutations. It is suggested that multiple active site mutations can cause significant structural changes of the active site which may contribute to these synergistic effects (37). Through recombining the genes of selected mutants, advantageous mutations are brought together and deleterious mutations can be separated out in random chimeras.

One of the key technologies of recombination is DNA shuffling, which is a breakthrough in directed evolution. It is an effective way to recombine homologous sequences with high identity (usually > 70%) and is straightforward to perform (29). In general, gene fragments are generated by digestion of the source DNAs and then are assembled into the full-length gene through PCR reactions. There are a number of other techniques available with their own characteristics such as the staggered extension process (StEP) (2) and random chimeragenesis on transient templates (RACHITT) (12). Moreover, incremental truncation for the creation of hybrid enzymes (ITCHY) was developed to recombine genes with low sequence similarity which is based on the truncation and ligation of DNA fragments (26).

Overall, the most commonly followed strategy of directed evolution experiments is the combination of error-prone PCR followed by shuffling of selected mutants with improved properties. The beneficial mutations contributing to the improved properties will be brought together through shuffling, and potential deleterious mutations of other mutants will not be shuffled to avoid the abolishment of the activity. If less bias or more efficient libraries are required in specific experiments, improved or modified techniques may be employed. Moreover, one of the most productive approaches of enzyme optimization can be a combination of rational design and directed evolution in the final analysis (10).

CHAPTER II

METHODOLOGY OF RANDOM MUTAGENESIS

Introduction

Directed evolution combines two essential components: methods for library construction and strategies for screening or selection for improved variants. Error-prone PCR is an effective method to construct a library of protein mutants. A successful screening design can be performed easily with high-throughput technologies and reflects accurately the requirements of an industrial application (35).

In this chapter, I describe a method to generate mutants of CynD and the strategy employed to screen the mutants for those with improved properties. Different error-prone PCR conditions were used to obtain an optimal mutation frequency. The screening system for improved mutants is based on a micro-plate assay that changes in color (red → yellow) upon hydrolysis of cyanide. We developed a high throughput two-dimensional screening strategy that was carried out at two different pH values.

Materials and methods

Culture media and reagents

All strains were grown in LB broth unless otherwise specifically mentioned. Antibiotics were used at the following concentrations: 100 µg/ml of ampicillin, 30 µg/ml of chloramphenicol, and 25 µg/ml of kanamycin. 5-Bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) was used at a concentration of 40 µg/ml. To induce protein

expression, a final concentration of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) was added to the cell culture.

Restriction enzymes, *Taq* DNA polymerase, T4 ligase, and antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). Bio-X-Short mix was purchased from Bioline (Randolph, MA). *Pfu* polymerase was purchased from Stratagene (La Jolla, CA).

Bacterial strains and plasmids

E.coli strain MB3436 (Δ*endA thiA hsdR17 supE44 lacI*^{q}*ZΔM15*) was used for routine cloning. Plasmid pBS (KS+) from Stratagene was the vector for subclonings. p2890 (17) is pET26b carrying $CynD_{pum}$ as a *Ned*I-*Xho*I fragment.

Error-prone PCR (epPCR)

Error-prone PCR was performed using the cloned full-length cyanide dihydratase gene as template to induce random mutations. The gene was amplified using mutagenic PCR conditions with primers T7 promoter primer (5' TAATACGACTCACTATAGGG 3') and T7 terminator primer (5' GCTAGTTATTGCTCAGCGG 3') which are vector primers outside the cloning sites. The PCR reaction mix consisted of 1×ThermoPol Reaction buffer, 200 µM of dNTPs, 2.5 U/50 µl of *Taq* DNA polymerase, 100 ng/50 µl of primers. Different concentrations of the DNA template (p2890) and $MnCl_2$ were used to get the optimal mutation frequency (3-5base pairs/kb gene). The PCR product was verified for correct size on an agarose gel and purified using SpinPrep® PCR clean-up Kit (Novagen, San Diego, CA).

Ligation of the PCR products

The purified PCR product was digested with *Xba*I and *Xho*I and ligated to the corresponding sites of pBS (KS+). Different conditions were employed to get the optimal ligation efficiency. The ligated DNA was electroporated into MB3436 and spread onto LB ampicillin agar plates with X-gal/IPTG. The recombinant plasmids were picked through blue/white colonies screening and the white colonies were subjected to further screening as described below.

Screening for alkali-tolerant mutants

A screening strategy was developed to test the cyanide degrading activity of the mutants at pH8 and pH10. The white colonies were manually picked into 96-well plates containing 150 µl LB broth with ampicillin and cultured at 37^{o}C overnight. The plates were copied onto LB-agar plates using a 48-tooth pronger (Boekel®, Feasterville, PA), grown and stored at 4^{o}C to save copies of the active clones. To test the activity of the mutants at pH8, 50 µl of cell culture was transferred into the corresponding wells of a 96-well plate. 50 µl of Tris buffer (100 mM, pH8) containing 8 mM of KCN was added and the 96-well plates were sealed with the parafilm and incubated at room temperature for 1 hour. The reactions were terminated by adding 100 µl of picric acid reagent (0.5% picric acid in 0.25 M sodium carbonate). The resulting mixtures were incubated at 65^{o}C for 15 minutes to allow color development and the active clones were identified by their yellow color. To test the cyanide-degrading activity of the mutants at pH10, the same conditions were used as that of pH8 except that 40 µl cells were mixed with 60 µl of Tris buffer (100 mM, pH10) containing 6.7 mM of KCN.

Results

Optimal error-prone PCR conditions

Different PCR conditions were adjusted to get the optimal mutation frequency, including the concentration of DNA template, $MnCl_2$, and the number of amplification cycles. A desired mutation frequency is three to five nucleotides per kb that yields one to five amino acid changes for the protein of interest.

Initially, different concentrations of DNA template were used with a fixed concentration of $MnCl_2$ and number of amplification cycles to roughly get the mutation frequency of the system. The mutant library was screened at pH8 to determine the frequency of mutants that lost activity, and about 40 clones from the library were randomly chosen from both the active and inactive groups for sequencing to reveal the mutation frequency (Table 1). All the sequenced active clones were tested for cyanide-degrading activity at pH10 in microfuge tubes and none showed activity.

TABLE 1. Mutation frequency at different concentrations of DNA template

Amount of DNA Template* (in 50 ul reaction)	**Number of Strains Screened**	**% Inactive Strains+**	**Mutation Frequency in Active Strains**	**Mutation Frequency in Inactive Strains**
15 ng	80	95%	0 (600 bp)	25 /kb
20 ng	90	98.9%	3/ kb	20 /kb
60 ng	160	90.6%	0.5 /kb	8 /kb
100 ng	187	94.1%	0.3/ kb	12.5 /kb
150 ng	140	88.6%	1 /kb	

*Conditions of the reaction: *Taq* DNA polymerase, $MnCl_2$: 0.4mM, amplification cycles: 35
+The cyanide-degrading activity was tested at pH 8.

From this data, the mutation frequency can be estimated from the frequency of inactive clones, and about 50% of inactive clones seemed desirable. Different PCR conditions were performed to obtain this frequency, adjusting the concentration of DNA template, $MnCl_2$, and the number of amplification cycles. After several rounds of testing, the error-prone PCR conditions was optimized at 130ng of target DNA template in a 50 μl reaction, 0.2 mM of $MnCl_2$, and 20 cycles of amplification.

Ligation condition optimization

The error-prone PCR products were digested and ligated to pBS (KS+). In order to get high ligation efficiency, the following additional steps were performed: the digested vector was treated with Antarctic phosphatase, ligated at 16^{o}C overnight, and the resulting ligation mixture was ethanol precipitated before electroporation.

Screening for mutants with improved activity

A high throughput screening system was developed based on a micro-plate assay that changes in color (red→yellow) upon hydrolysis of cyanide. The wild-type control strains can degrade cyanide at pH8 but not at pH10. To screen for mutants able to degrade cyanide at higher pH, clones of the mutant library generated by error-prone PCR were tested in 96-well plates at pH10. The activity of the candidates was reconfirmed by repeating the reaction under more controlled conditions in microfuge tubes to eliminate false positive results.

Two candidate clones (mutants C5 and H7) that were able to degrade cyanide at pH10 were found from approximately 2,000 clones that were screened. The DNAs of these mutants were sequenced to identify the nucleotide changes.

Discussion

Directed evolution is a powerful tool to improve the properties of proteins. The approach has the advantage that detailed knowledge about the relationship between molecular structure and function is not required. The methodology of directed evolution usually comprises the creation of a pool of mutated genes and subsequent screening for improved gene products; this process is then iterated for 2 or 3 cycles. In this study, I developed a method to generate CynD mutants and a strategy to screen the mutants for improved activity at higher pH.

Error-prone PCR was employed to construct a library of protein mutants. First, the optimal mutation frequency to construct a library of mutants needed to be determined. A strategy was developed to determine the mutation frequency without sequencing. The mutation frequency could be deduced from the frequency of inactive clones. When the other PCR conditions were fixed and only the concentration of the DNA template was adjusted, the mutation frequencies of the active clones and inactive clones showed large differences (Table 1). The inactive group had high mutation frequencies from 8 to 25 mutations/kb as the amount of the DNA template decreased, while the active group had a mutation frequency from 0.3 to 3 mutations/kb. The active clones could not degrade cyanide at pH10. From the data, I concluded that high mutation frequency (>6 mutations/kb) would generally abolish the cyanide-degrading activity while low mutation frequency (<3 mutations/kb) usually won't contribute to the better properties of the enzyme. It should also be noted that with the high mutation frequency of the inactive group, the percentage of inactive strains of the mutant library is very high (between

88.6% to 95%, Table 1). Therefore, for an optimal mutation frequency of 3-5 mutations/kb, which lead to one to five amino acids changes in the target protein, the generated mutant library should have about 50% of clones that are inactive at pH8.

The error-prone PCR conditions to get the desired mutation frequency were optimized. The cyanide-degrading activity of the clones generated by error-prone PCR under each PCR condition was tested in micro-plates at pH8 and the percentage of inactive clones was obtained to estimate the mutation frequency. To get the optimal error-prone PCR conditions, I increased the amount of DNA template first but the mutation frequency was still too high. Then I tried to lower the concentration of $MnCl_2$ from 0.4 mM to 0.2 mM, the amplification cycles from 35 to 25 and 20 cycles, or use different amounts of target DNA. After several rounds of testing, the error-prone PCR conditions were optimized at 130ng of target DNA template in a 50 μl reaction, 0.2 mM of $MnCl_2$, and 20 cycles of amplification.

The third critical step to overcome is getting high ligation efficiency of the error-prone PCR products to the vector. The primary limitation of library size depends on the ligation efficiency of the PCR product into the vector and large libraries require considerable effort to optimize (33). I took several additional steps compared to the standard protocol of DNA ligation and electroporation: the digested vector was treated with phosphatase to eliminate the vector ligating to itself, the DNA was ligated at optimal temperature for longer time (16^oC overnight), and the ligated DNA was ethanol precipitated before electroporation to get higher electroporation efficiency.

A successful screening design can be performed easily with high-throughput technologies and reflects the needs of an industrial application (35). Since cyanide is usually maintained in solutions at pH9 or higher to avoid production of hydrogen cyanide gas, I choose to create an enzyme that can degrade cyanide at pH10 as an important improved property. I developed a high throughput two-dimensional screening strategy that was carried out at two different pH values. This screening system is based on a micro-plate assay that changes in color (red→yellow) upon hydrolysis of cyanide. At pH8, the cyanide-degrading activity of the clones was tested to identify positive clones and at pH10 the cyanide-degrading activity of the mutants was tested to screen for the alkali-tolerant mutants. To avoid false positive results, the test of the selected mutants was repeated in microfuge tubes under more controlled conditions.

It would be useful in the future to generate and screen more mutants under the optimal error-prone PCR condition at pH10. DNA shuffling could be employed with these mutants in order to bring beneficial mutations together. Through this method, an even more improved mutant might be obtained with synergistic effects of multiple mutation sites.

CHAPTER III

CHARACTERIZATION OF $CynD_{pum}$ MUTANTS C5 AND H7

Introduction

There is a potential demand for cyanide-degrading enzymes with better properties that can tolerate the adverse conditions of cyanide waste bioremediation in industry. These properties include higher pH tolerance, catalytic activity, thermal stability, or tolerance to solvent. These cyanide-containing wastes differ in pH and solute content because of their different industrial sources. Since these varied conditions have a significant impact on enzymatic action and the existing cyanide-degrading nitrilases all have their limits for use as agents in bioremediation under different conditions, engineering a cyanide-degrading enzyme with improved properties would be of great benefit to industrial cyanide remediation.

Higher pH tolerance of cyanide-degrading enzymes was chosen as an important property in this work. Cyanide volatilizes easily with a low boiling point of 25.7 ^{o}C (19). Solutions containing cyanide are usually stored at high pH to prevent volatilization which could produce hydrogen cyanide gas. A cyanide waste sample from an electroplating plant in Houston, TX was tested and found to be pH11 with 1M of cyanide. Therefore, the enzymes that could degrade cyanide at alkaline conditions would be good candidates for industrial cyanide remediation.

In this chapter, I describe the characterization of two $CynD_{pum}$ mutants of *B. pumilus* C1 with higher pH tolerance generated by random mutagenesis in Chapter II.

The mutants each have three different amino acid changes compared to the wild-type enzyme. The individual point mutants were constructed to study the effect of each amino acid residue on the *in vivo* and *in vitro* cyanide-degrading activities. From the previous studies, tolerance to pH might be related to the structural stability which can be measured by the thermal stability of the enzymes. Therefore, the pH activity profile and the thermal stability of the each purified enzyme were studied. The study of these mutants provides insight into the underlying mechanisms of catalysis or protein structure. This information will be employed for the construction of optimized enzymes.

Materials and methods

Culture media, reagents, bacterial strains and plasmids

All the culture media and reagents used in this chapter are the same as those in chapter II, unless described specifically. A detailed description of all the bacterial strains and plasmids used and constructed in this chapter is provided in Table 2.

Construction of single and double point mutants

Alleles of $CynD_{pum}$ carrying each of the six amino acid changes were constructed by site-directed mutagenesis. The mutagenesis reactions were carried out with mutagenic primers using the QuickChange® protocol (Stratagene, La Jolla, CA). Complementary primer pairs containing the desired DNA changes are listed in Table 3. 130ng of each primer, 100ng of dsDNA template, and 2.5U of Pfu polymerase were combined in a total reaction volume of 50 µl. Thermal cycling parameters were 95°C for 30 sec and 16 cycles of 95°C for 30 sec, 50°C for 1 min and 68°C for 5 min. Successful construction of

the mutations were confirmed by DNA sequencing and are summarized in Table 2. The double mutants were made similarly.

TABLE 2. Plasmids used and constructed carrying various $CynD_{pum}$ alleles

Strains and plasmids	Description	Reference
pBC (SK+)	Cam^r *E. coli* cloning vector	Stratagene
pBS (KS+)	Amp^r *E. coli* cloning vector	Stratagene
pET-28b	Kan^r *E. coli* expression vector	Novagen
p3287	pBS carrying $CynD_{pum}$ Δ (293-330)	Lab stock
p3817	pBS carrying $CynD_{pum}$ Q86R+ E96G + D254E (C5)	This work
p3818	pBS carrying $CynD_{pum}$ E35K+ Q322R+ E327G (H7)	This work
p3861	pBS carrying $CynD_{pum}$ Q86R	This work
p3862	pBS carrying $CynD_{pum}$ E96R	This work
p3863	pBS carrying $CynD_{pum}$ D254E	This work
p3870	pBS carrying $CynD_{pum}$ E35K	This work
p3871	pBS carrying $CynD_{pum}$ Q322R	This work
p3879	pBS carrying $CynD_{pum}$ E327G	This work
p3832	pBS carrying $CynD_{pum}$ Q322R+ E327G	This work
p3903	pBS carrying $CynD_{pum}$ Q86R+ E96R	This work
p3904	pBS carrying $CynD_{pum}$ Q86R+ D254E	This work
p3905	pBS carrying $CynD_{pum}$ E96R+ D254E	This work
p3927	p3817 with Q322R	This work
p3928	p3817 with E327G	This work
p3978	pBS carrying RBS and His6 of pET28	This work
p3979	p3978 carrying $CynD_{pum}$ Δ(293-330)	This work
p3980	p3978 carrying $CynD_{pum}$ C1	This work
p3981	p3978 carrying $CynD_{pum}$ Q86R+ E96G + D254E	This work
p3982	p3978 carrying $CynD_{pum}$ Q322R+ E327G	This work
p3983	pBC carrying $CynD_{pum\text{-}6His}$ Δ (293-330)	This work
p3984	pBC carrying $CynD_{pum\text{-}6His}$	This work
p3986	pBC carrying $CynD_{pum\text{-}6His}$ Q86R+ E96G + D254E	This work
p3988	pBC carrying $CynD_{pum\text{-}6His}$Q322R+ E327G	This work
p4005	pBC carrying $CynD_{pum\text{-}6His}$ E35K+ Q322R+ E327G	This work
p4007	pBC carrying $CynD_{pum\text{-}6His}$ E327G	This work
p4024	pBC carrying $CynD_{pum\text{-}6His}$ Q322R	This work
p4025	pBC carrying $CynD_{pum\text{-}6His}$ Q86R+ E96R	This work
p4026	pBC carrying $CynD_{pum\text{-}6His}$ Q86R+ D254E	This work
p4027	pBC carrying $CynD_{pum\text{-}6His}$ E96R+ D254E	This work
p4045	pBC carrying $CynD_{pum\text{-}6His}$ Q86R+ E96G + D254E+ E327G	This work

TABLE 3. Primer sequences used in site-mutagenesis*

Primer	Sequence
Q86R	5‘CCCTAGCTTAGCCATTCGCAAAATAAGTGAGGCAGC3’ 5'GCTGCCTCACTTATTTTGCGAATGGCTAAGCTAGGG3'
E96G	5'GGCAGCAAAGAGAAATGGAACGTACGTTTG3' 5'CAAACGTACGTTCCATTTCTCTTTGCTGCC3'
D254E	5'GGAACCGATCAGTGAAATGGTTCCAGCTGAAACAG3' 5'CTGTTTCAGCTGGAACCATTTCACTGATCGGTTCC3'
E35K	5’GTGAACTGATCGACAAGGCAGCTTCAAATGG3’ 5’CCATTTGAAGCTGCCTTGTCGATCAGTTCAC5’
Q322R	5’GGACATTCAATATCGCCATGGTATACTGG3’ 5’CCAGTATACCATGGCGATATTGAATGTCC3’
E327G	5’CATGGTATACTGGGAGAAAAAGTTTAAACGG3’ 5’CCGTTTAAACTTTTTCTCCCAGTATACCATG5’

*: The underlined nucleotides correspond to the substituted bases.

Expression and purification of the wide-type and mutated $CynD_{pum}$

The pBS-HisTag-CynDΔ vector (p3979) was constructed in two steps: a DNA fragment containing the ribosome binding site and six-histidine tag of pET28 was generated with *Xba*I and *Xho*I and inserted into pBS (KS+) vector to construct p3978; p3287 (pBS carring an inactive $CynD_{pum}$ with the C-terminal deletion Δ293-330) was digested with *Nde*I and *Xho*I and cloned into p3978 to generate p3979. The DNA of the wild-type and mutant $CynD_{pum}$ were excised by H*ind*III and *Xho*I digestion of the plasmids above and subcloned into p3979 to add a polyhistidine tag to the N-terminus of each protein (the constructs are listed in Table 2).

The His6 fusion constructs were all digested with *Xba*I and *Xho*I and subcloned to pBC (SK+) vector. This inverts the gene to allow expression from the *lac* promoter. All

the resultant subclones were transferred to MB3436 *E. coli* cells and are summarized in Table 2.

The wild-type $CynD_{pum}$ and its mutants, all with a His tag, were purified using a Ni Sepharose High Performance column (HisTrap™, Amersham Biosciences, Piscataway, NJ). Each plasmid containing strain was cultured at 37°C overnight in 50ml of LB broth containing chloramphenical and IPTG. The cell cultures were centrifuged at 3,750rpm and 4°C for 15min and resuspended in 20ml solution containing 0.1 M NaCl, 20 mM sodium phosphate (pH7.4), 12.5 mM imidazole (pH7.4). Cell lysis was performed by adding 1mg/ml lysozyme with incubation on ice for 15 min and freezing and thawing 5 times. At the last thaw, 100 µl *Serratia* nuclease was added to remove nucleic acid and the cells were centrifuged at 3,750rpm and 4°C for 15min to collect the supernatants. The supernatants containing the soluble $CynD_{pum}$ were loaded onto a 1-ml HisTrap™ column which had been washed with 10 column volumes of binding buffer containing 100 mM NaCl, 20 mM sodium phosphate, and 25 mM imidazole. After passing the samples through the HisTrap column, it was washed with 10 column volumes of washing buffer (binding buffer plus 100 mM imidazole). The his-tagged enzymes were eluted with the elution buffer (binding buffer plus 500 mM imidazole).

The purified proteins were quantified by the Bradford method (Amresco®, Solon, OH) with bovine serum albumin as the standard. The purity and molecular weight of the purified proteins were confirmed by SDS-PAGE with Coomassie Brilliant Blue staining.

SDS-PAGE

To confirm the purity of the purified proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. 5 µl samples of eluates was mixed with 10 µl loading buffer (ICC Bioexpress®) and boiled for 10 min. The prepared protein samples were separated on 10% polyacrylamide gradient gels and stained with coomassie blue.

pH activity profiles

Activities of the purified enzymes were measured at pHs ranging from 7.0 to 10.0 under standardized assay conditions using the following buffers: 100 mM sodium phosphate for pH7.0 and 8.0; 500 mM sodium phosphate for pH8.5, 9.0, 9.5, and 10.0. Reactions were run at room temperature and for up to 61 minutes. Samples were taken at 1 minute and the other time points when a measurable amount of cyanide still remained. The picric acid endpoint method was used to determine cyanide concentration (Fisher and Brown, 1952). For each pH condition, 1 M of KCN stock solution was diluted to a final concentration of 5 mM to start the reactions. At each time point, 100 µl aliquots were removed and 200 µl picric acid reagent (0.5% picric acid in 0.25M sodium carbonate) was added to terminate the reaction. The resultant mixtures were boiled for 6 min to allow color development and the absorbance was read at 520nm. The rate of degradation was represented by the change in absorbance per minute. Enzyme concentrations used for rate measurements were: 8.88µg ml^{-1} for $CynD_{pum}$ at pH7.0 and pH8, 14.8µg ml^{-1} for $CynD_{pum}$ at pH8.5 and pH9.0 and 16 times more enzyme used for

pH9.0, pH9.5 and pH10.0; 7.98μg ml^{-1} for mutant C5 at pH7.0 and pH8, 13.3μg ml^{-1} for mutant C5 at pH8.5 and pH9.0, and 16 times more enzyme used for pH9.5 and pH10.0.

Thermal stability

To determine the thermal stability, the purified enzymes diluted in 100 mM MOPS (pH7.6) were incubated at 42^{o}C and the samples were taken at time points ranging from 0 min to 72 h. Enzyme concentrations used for rate measurements were: 8.88μg ml^{-1} for $CynD_{pum}$ and 7.98μg ml^{-1} for mutant C5. The activities were measured at room temperature as described in pH activity profiles except using 100 mM MOPS buffer pH7.6.

Results

Sequence analysis of C5 and H7 mutant genes

DNA sequencing of plasmids C5 and H7 revealed that there are three amino acid changes in each mutant compared to the wild-type *cynD* gene. Mutant C5 carries the mutations Q86R, E96G, and D254E as well as two silent changes (A705G and A981G). Mutant H7 has the mutations E35K, Q322R, and E327G and one silent mutation (A816G). All the nucleotides substitution and amino acids changes are highlighted in color in the wild-type *cynD* sequence shown below (Fig. 2).

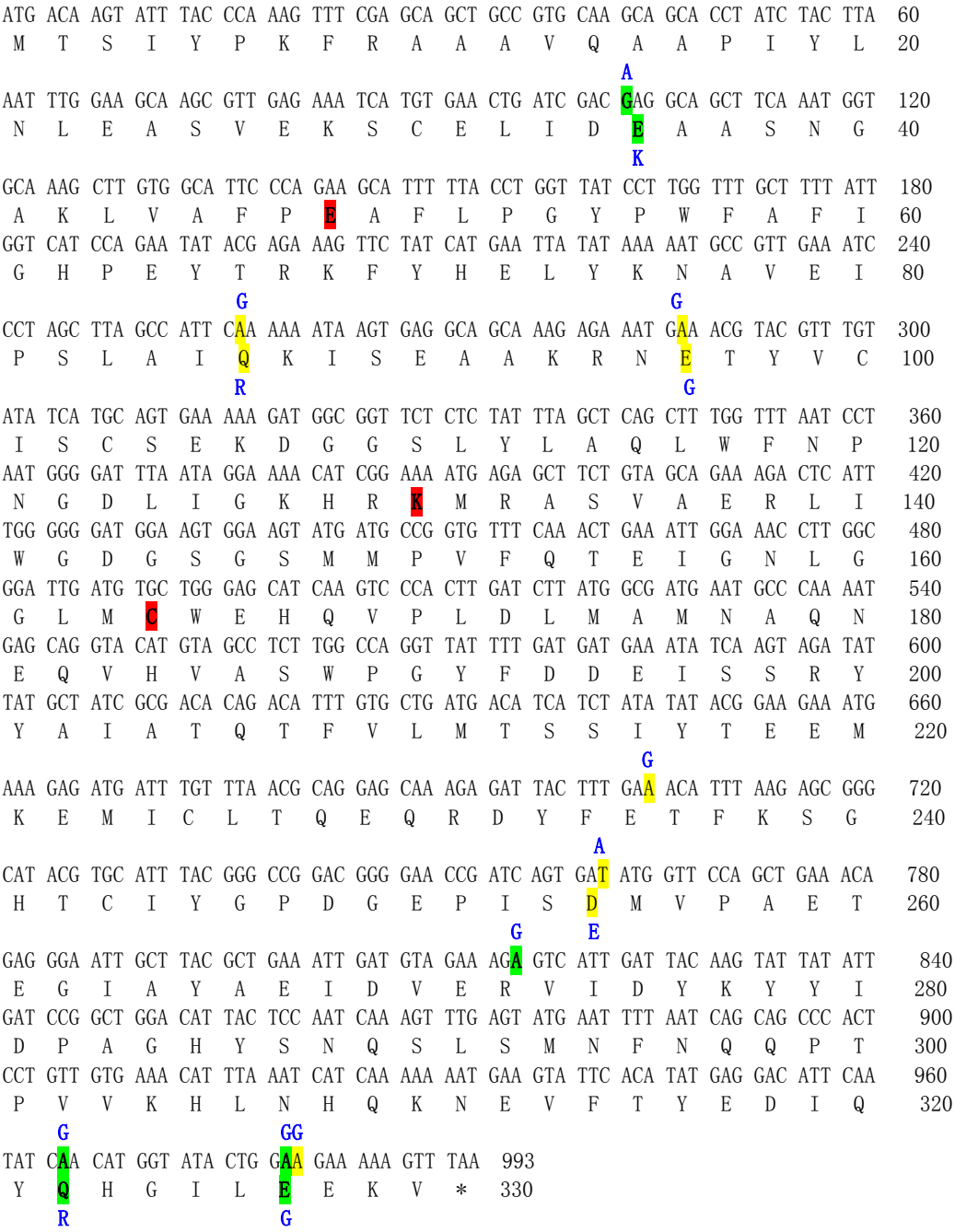

FIG. 2. DNA and amino acid changes of mutant C5 and H7.
The residues in yellow color showed the nucleotides and amino acids changes of mutant C5.
The residues in green color showed the nucleotides and amino acids changes of mutant H7.
The residues in red color showed the conserved Glu-Lys-Cys catalytic site

In vivo activity of $CynD_{pum}$ mutants in *E. coli*

The activity in cultures of C5, H7, and the single and double mutants were tested at pH8 and pH10 (Table 4). For mutant C5, the single and double mutants had significantly less activity at pH10 than the original mutant C5. For mutant H7, the activity of E327G mutant was the same as the original mutant H7; E35K did not degrade cyanide at pH10; the activity of Q322R mutant was intermediate at pH10.

TABLE 4. Cyanide-degrading activity of the mutant alleles

Strains	**Activity**		**Strains**	**Activity**	
	pH8	**pH10**		**pH8**	**pH10**
$CynD_{pum}$ wt	+++	-	E35K+Q322R+E327G (H7)	+++	+++
Q86R+E96G+D254E (C5)	+++	+++	E35K	+++	-
Q86R	+++	+(+)	Q322R+E327G	+++	+++
E96G	+++	+	Q322R	+++	+(+)
D254E	++(+)	-	E327G	+++	+++
Q86R+E96G	+++	++	C5+ Q322R	+++	+++
Q86R+D254E	+++	+(+)	C5+ E327G	+++	+++
E96G+D254E	++(+)	+/-			

The activity of the mutants was tested under the screening condition as described in Materials and Methods in Chapter II: cell culture of the mutants was mixed with Tris buffer (100mM, pH8 and pH10) with a final concentration of cyanide being 4mM; the assay was performed in centrifuge tubes.
+: represents relative cyanide-degrading activity; -: no cyanide-degrading activity.

SDS-PAGE

To confirm the purity of the His-tagged proteins purified by Ni Sepharose column, SDS-PAGE was performed. As can be seen in Fig. 3, the protein is homogeneous.

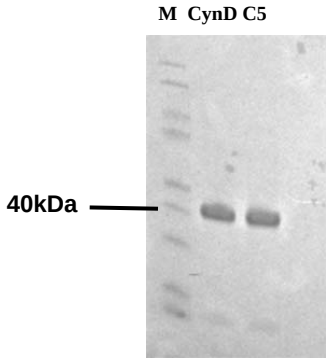

FIG. 3. SDS-PAGE of purified $CynD_{pum}$ and mutant C5.
The purified protein were observed after coomassie brilliant blue staining of the gel

pH tolerance of $CynD_{pum}$ mutants

The pH activity profile of the purified enzymes was measured (Table 5). The activity of the wild-type enzyme and mutant C5 are similar at pH7.0 and pH8.0. To measure the activity at pH8.5 or higher, the concentration of the enzymes was increased. At pH9.0, the activity of mutant C5 was the same as at the lower pH. In contrast, there was no detectable cyanide-degrading activity by the wild-type enzyme at pH9.0, despite 16-fold more enzyme and a longer reaction time (8 hours). No activity was detected at pH9.5 and pH10.0 for either purified wild-type enzyme or mutant C5 at the same higher enzyme concentration and increased time.

TABLE 5. The pH profile of purified enzymes

pH value	Δ OD/min (1μg ml^{-1} enzyme) ×100	
	Wild-type	**Mutant C5**
7.0	3.38	3.76
8.0	7.66	7.77
8.5	17.84	19.25
9.0	≤ 0.113	8.95
9.5	≤ 0.026	≤ 0.027
10.0	≤ 0.003	≤ 0.004

The cyanide-degrading activity of the enzymes was tested as described in Materials and Methods.
The rate of degradation was represented by the change in absorbance per minute per μg ml^{-1} enzyme ×100.

Thermal stability of $CynD_{pum}$ mutants

In a previous thermal stability study of $CynD_{pum}$, the stability of this enzyme was tested by incubating the enzyme at 23°C, 37°C, 42°C, and 55°C (18). That work showed that 42°C was an appropriate condition to test thermal stability. To compare the temperature stability of the wild-type enzyme and mutants, each enzyme was incubated at 42°C for time periods range between 0 and 72 hours and their relative residual activities were measured (Table 6 and Fig. 4). For the wild-type enzyme, 60% of the activity was lost by 24 hours and 80% of the activity was lost after 72 hours. For mutant C5, 40% of the activity was lost by 24 hours and about 60% of the activity was lost after 72 hours. It should also be noted that mutant C5 still retained 88% activity after 8 hours, twice that of the wild-type enzyme. Therefore, mutant C5 is more stable than the wild-type enzyme.

TABLE 6. Stability of mutant alleles at 42°C

Incubation time (hours)	Wild-type		Mutant C5	
	Δ OD/min	Relative activity (%)	Δ OD/min	Relative activity (%)
0	0.0099	100	0.0094	100
4	0.0062	63	0.0088	94
8	0.0042	43	0.0083	88
24	0.0038	39	0.0062	66
48	0.0021	21	0.0054	58
72	0.0023	23	0.0035	37

The cyanide-degrading activity of the enzymes was tested as described in Materials and Methods.
The data in the table is the average from duplicate experiments.

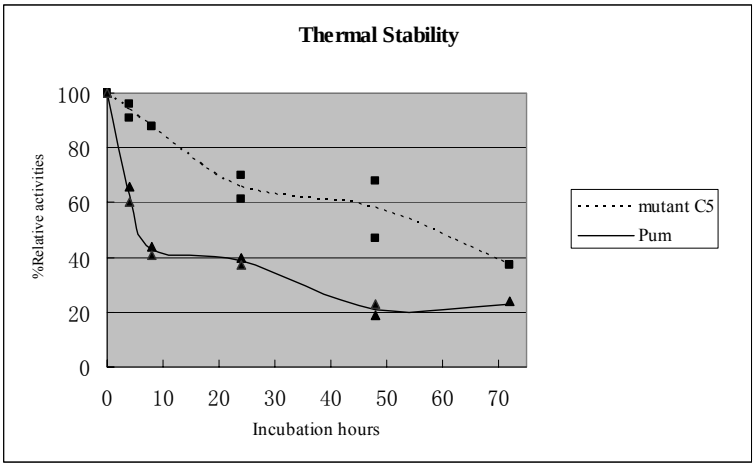

FIG. 4. The thermal stabilities of the wild-type enzyme and mutant C5.
Residual activities were tested after incubation at the labeled time periods**.**
Symbols represent values from duplicate experiments with the *line* as the average

Discussion

The characteristics of two selected cyanide dihydratase mutants which I described in chapter II were studied and compared to that of the wild-type enzyme. Both mutants have three amino acids changes from the wild-type enzyme. Alleles were also constructed to study each single amino acid change. The cyanide-degrading activities of

these mutants were tested. The pH activity profiles and thermal stability of each purified protein were measured.

From the *in vivo* cyanide-degrading activity test (Table 4), $CynD_{pum}$ mutants C5 and H7 can degrade cyanide at pH10 while the wild-type $CynD_{pum}$ can not. The single and double point mutants of C5 were less active at pH10 than the parental mutant C5. This result may suggest that the mutations Q86R, E96G, and D254E all contribute to the increased activity of mutant C5 at high pH. In contrast, with mutant H7, the allele E327G behaved similar to the parental H7. The E35K allele did not degrade cyanide at pH10, and the Q322R allele showed less activity at pH10. This suggests that E327G is the major contributor to the H7 phenotype.

The pH activity profile of the purified enzymes was also measured (Table 5). The activity of the wild-type and mutant C5 enzyme are similar at pH7.0 and pH8.0. The mutant C5 was similarly active at pH9.0, but not the wild-type $CynD_{pum}$. Compared to the *in vivo* activity, the purified mutant C5 could not degrade cyanide at pH9.5 or higher even with increasing concentration (16-fold) of enzyme or longer reaction time (8 hours). The purified enzymes might be more sensitive to different pH conditions compared to the enzymes in cells; presumably the cells can partially correct for the increased external pH.

There are two different mechanisms that could explain the increased pH tolerance. One would be an improved capacity of the enzyme to catalyze the reaction at higher pH. The second is the enzyme, normally unstable at pH9 or higher, is now stabilized at these higher pHs. The thermal stability of the purified wild-type $CynD_{pum}$ and the mutants

were measured to study if these mutations increased the stability of the enzyme. The thermal stability of mutant C5 was greatly enhanced at 42^{o}C. It had about twice the activity of the wild-type enzyme after 8 and 48 hour incubation (Table 6, Fig. 4).

A crystal structure of any microbial nitrilase, including cyanide dihydratase, still remains elusive. Previous studies suggest that oligomer formation is essential for the activity of these enzymes and the C-terminal extension contributes to the formation and/or stabilization of the oligomeric quaternary structure (31, 32). The three amino acid changes of mutant C5 (Q86R, E96G, and D254E) are spread around the conserved Glu48-Lys130-Cys163 catalytic site (Fig. 2). The mutations E96G and D254E are also charge changes. However, without an accurate structure it is hard to determine whether they might affect the catalytic domain.

The key amino acid change E327G of mutant H7 is located in the C-terminal extension (Fig. 2). There is a charge change in mutation E327G as well. Previous studies demonstrated the effects of the C-terminus to the activity of $CynD_{pum}$. The structure of $CynD_{pum}$ switched from an octadecamer at pH8 to a long helical fiber at pH5.4 and this change of quaternary structure may be due to the histidine residues in its C-terminus (17). An increase in this enzyme's activity at this pH5.4 inflection point was also observed compared to other cyanide-degrading enzymes (18). This activity change was due to the number of the available subunits. $CynD_{pum}$ was shown to tolerate the C-terminal truncations back to residue 293 before the activity was lost (32). $CynD_{pum}$ also functioned with C-terminus of $CynD_{stut}$ while $CynD_{stut}$ cannot function with the C-terminus of $CynD_{pum}$ (32). Mutation E327G is located in the C-terminal region without

which $CynD_{pum}$ can still function. However, allele E327G showed increased activity at higher pH and behaved similarly to parental mutant H7. From the above effects of the C-terminus and this study, E327G might increase the stabilization of the spiral structure at higher pH.

In the future, screening more clones under the optimal error-prone PCR conditions might generate other interesting mutations. Having more mutants with improved properties, DNA shuffling could be employed to synergize their effects and produce enzymes that tolerate the adverse conditions of industrial applications.

Overall, this work demonstrated the successful improvement of a cyanide dihydratase by increasing activity at alkaline pH through random mutagenesis for the first time. None of the amino acid mutations found in mutant C5 or H7 have been previously described to have an influence on the pH activity profile or thermal stability.

CHAPTER IV

PROBING THE SUBSTRATE SPECIFICITY OF A NITRILASE

Introduction

The nitrilases (E.C.3.5.5.1) are the only branch of the nitrilase superfamily that can convert nitriles (RCN) to the corresponding acids and ammonia. They hydrolyze a variety of nonpeptide carbon-nitrogen bonds utilizing a characteristic Glu-Lys-Cys catalytic triad (8). Nitrilases use a broad range of nitriles as substrates and have important applications to produce a variety of fine chemicals for the pharmaceutical and chemical industry (6). Most of the known enzymes are found from bacteria, fungi, and plants. The microbial nitrilases include nitrilase, cyanide hydratase and cyanide dihydratase.

The *Bacillus* sp. OxB-1 nitrilase shows a high level of identity with the cyanide dihydratases from *B. pumilus* C1 and *P. stutzeri* AK61. These enzymes are similar at the amino acid level with an identity of 72.8% (Fig. 5), but they recognize different substrates. The nitrilase of *Bacillus* sp. strain OxB-1 hydrolyzes phenylacetonitrile to phenylacetic acid in aldoxime metabolism (20). Cyanide dihydratases use cyanide as the only substrate. Their overall similarity provides a good model to study the substrate specificity for cyanide recognition and the substrate specificity of nitrilases in general.

The goal of this study is to convert the nitrilase of OxB-1 so that it can degrade cyanide and thereby obtain a more complete understanding of substrate recognition by cyanide degrading enzymes.

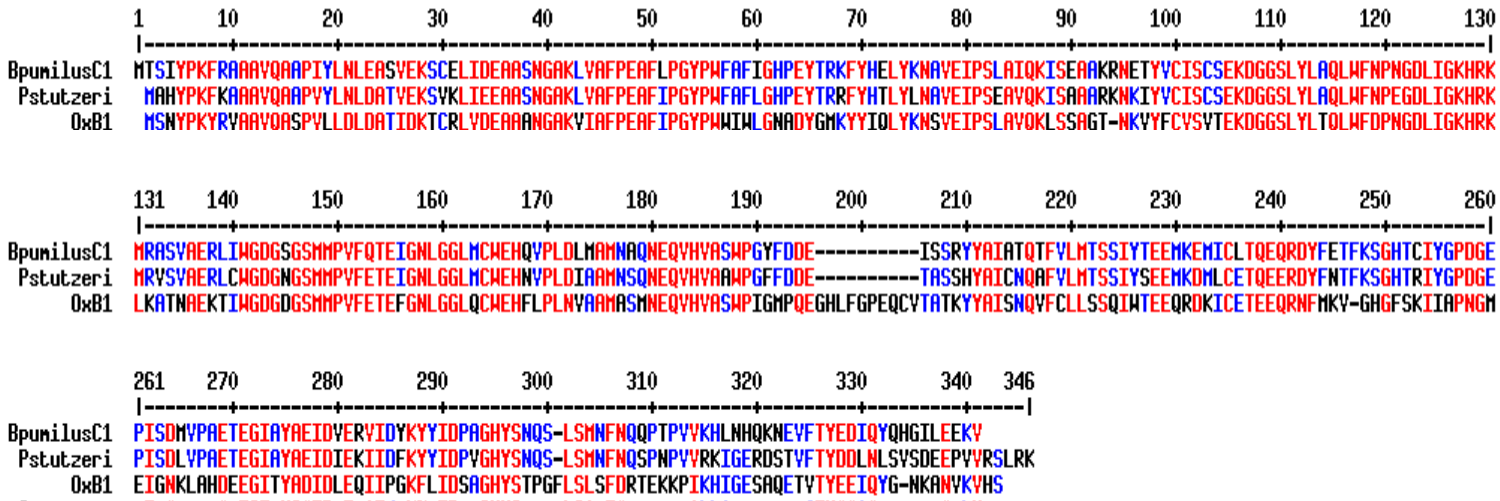

FIG. 5. Multiple protein sequence alignment of the OxB-1 nitrilase with cyanide dihydratases.
The aa in red color are similar in all 3, in blue are similar for 2 of 3 sequences, and in black are not conserved

Materials and methods

Culture media, reagents, bacterial strains and plasmids

All the culture media and reagents used in this chapter are the same as those in Chapter II, unless otherwise mentioned specifically. A detail description of all the bacterial strains and plasmids used and constructed in this chapter is provided in Table 7. The sequences of the primers used to construct the plasmids in this chapter are summarized in Table 8.

TABLE 7. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
pBS(KS+)	Amp^r *E.coli* cloning vector	Stratagene
pET-26b	Kan^r *E.coli* expression vector	Novagen
p2890	pET26b carrying $CynD_{pum}$	Lab stock
p2935	pBS(KS+) carrying OxB-1 nitrilase (deletion of A at 344)	Lab stock
p3504	pBS(KS+) carrying $CynD_{pum}$	Lab stock
p3625	pET26b carrying OxB-1 nitrilase	This work
p3729	pET26b carrying OxB-1 deletion of 192-204 (OxBΔ1)	This work
p3730	pBS(KS+) carrying OxBΔ1 (1-218)-$CynD_{pum}$(223-330)	This work
p3736	pBS(KS+) carrying $CynD_{pum}$(1-223)-OxBΔ1(232-330)	This work
p3864	pBS(KS+) carrying OxB-1 (1-145)- $CynD_{pum}$ (147-330)	This work
p3866	pBS(KS+) carrying $CynD_{pum}$ (1-147)-OxBΔ1(146-330)	This work

TABLE 8. PCR primers used for plasmid construction

Primers	Used to make plasmid
wt-F: 5'GCGGACTACGGTATGAAATACTACATCCAGCTGTAC3' wt-R: 5'GTACAGCTGGATGTAGTATTTCATACCGTAGTCCGC3'	p3625
delta-F: 5'GGCCGATCGGTATGGACGATGAAACCGCGACCAAATAC3' delta-R: 5'GTATTTGGTCGCGGTTTCATCGTCCATACCGATCGGCC 3'	p3729
OxB-R1:5'CCTGCGTTAAACAAATCATGTCACGCTGTTCTTCGGTCC 3' C1-F1:5'GGACCGAAGAACAGCGTGACATGATTTGTTTAACGCAGG 3'	p3730
C1-R2: 5'CTTCGGTTTCGCAGATTTTCTCTTTCATTTCTTCCG3' OxB1-F2: 5'CGGAAGAAATGAAAGAGAAAATCTGCGAAACCGAAG3'	p3736
OxB1-R3:5'GTTTGAAACACCGGCATCATAGAACCGTCACCGTCACC3' C1-F3:5'GGTGACGGTGACGGTTCTATGATGCCGGTGTTTCAAAC3'	p3864
OxB-F4:5'GGGGATGGAAGTGGAAGTATGATGCCGGTTTTCGAAACCG3' C1-R4:5'CGGTTTCGAAAACCGGCATCATACTTCCACTTCCATCCCC3'	p3866

Construct wild type *Bacillus* sp. OxB-1 nitrilase gene

To get the wild- type OxB-1 nitrilase gene, Quickchange site-directed mutagenesis was performed to correct a frameshift mutation of p2935 (the synthetic OxB-1 nitrilase gene constructed in the lab). The primers wt-F and wt-R were used (Table 8). The amplification conditions were 95^{o}C for 30 sec, 12 cycles of 95^{o}C for 30 sec, 55^{o}C for 1 min and 68^{o}C for 4 min. The resulting product was confirmed by sequencing.

Gene expression

The corrected OxB-1 nitrilase gene was cloned into pET26b to generate p3625 after digestion of both vector and insert with *Nde*I and *Xho*I. The ligation products were transformed into *E. coli* MB3436 and selected on LB kanamycin medium. Transformants were analyzed by restriction digestion with *Xba*I and *Xho*I.

For expression, p3625 was transformed into *E.coli* BL21, a 1.5 ml culture was started using 1/100 volume of an overnight culture and grown for 2 hours, the cells were induced by the addition of IPTG to a final concentration of 1 mM and further grown for 2 hours.

SDS-PAGE and Westen blot

To confirm expression of the OxB-1 nitrilase, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The cells were harvested by centrifugation at 5,000 rpm for 1min. Cell pellets were resuspended in 1 ml of 1M Tris HCl and sonicated 10 cycles for 20 seconds at 10- second intervals. 5 µl cell lysate sample was mixed with 10 µl loading buffer (ISC Bioexpress®) and boiled for 10 min. The protein samples were separated on 10% polyacrylamide gradient gels.

The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (PIERCE, Rockford, IL) with the Genie Blotter (IDEA, Minneapolis, MN). Polyclonal rabbit antiserum raised against a mixture of cyanide hydratase and cyanide dihydratase proteins was used as the primary antibody at 1:2000 dilution. For immunoblotting, Western MAX Horseradish Peroxidase Kit (AMRESCO, Solon, OH) was used. HRP conjugated goat anti-IgG secondary antibody was used at 1:5000 dilution and reacted with DAB (3,3'-Diaminobenzidine) substrate for detection.

Construction of OxB-1Δ(192-204)

To delete the 13 amino acid region (192-204) in OxB-1 nitrilase that CynD does not have, Quickchange site-directed mutagenesis was performed. Thermal cycling

parameters were 95°C for 30 sec, 18 cycles of 95°C for 30 sec, 55°C for 1 min and 68°C for 6 min 30 sec with primer delta-F and delta-R (Table 8).

The mutants were sequenced to confirm the deletion and stocked as MB3729. To test expression, the plasmid was transformed to *E.coli* BL21.

Construction of hybrid proteins

The hybrid proteins were constructed in this general way. First, two separate PCR reactions were performed to get each gene segment from OxB-1 nitrilase and CynD of *pumilus* C1 separately. The primers were designed with about 20 base pairs overlapping to allow annealing of the two generated PCR fragments in the subsequent PCR reaction. Then, the two PCR products were combined as the DNA template to get the full-length gene of the hybrid protein using the outermost primers. Four hybrid proteins were constructed in this way and the description of each is listed in Table 7.

To construct the hybrid clone p3730, two separate PCR reactions were performed first. p3729 was used as template in one PCR reaction with the primers OxB-R1 (Table 8) and T7 promoter primer (sequence provided in Chapter II, Materials and methods) to get OxBΔ1 segment encoding residues 1-218. p2890 was used as template in another PCR reaction with the primers C1-F1 (Table 8) and T7 terminator primer (sequence provided in Chapter II, Materials and methods) to get $CynD_{pum}$ segment encoding residues 223-330. Primers OxB-R1 and C1-F1 have a 19-base pair overlapping. BIO-X-ACT short mix (Bioline, Randolph, MA) was employed for both amplifications. The amplifications ran at 95°C for 2 min, 5 cycles of 95°C for 30 sec, 45 °C for 1 min and 68 °C for 1 min,

25 cycles of 95 °C for 30 sec, 55 °C for 1 min and 68 °C for 1 min, and a final extension of 68 °C for 5 min.

To generate the full-length hybrid gene of p3730, the two PCR fragments were purified from a 0.7% agarose gel and combined at a 1:1 ratio as the DNA template. To allow the two fragments of the DNA template to anneal well and elongate, the DNA template and PCR buffer mixture was treated at 95°C for 2 min, with 10 cycles of 95°C for 30 sec, 50°C for 30 sec and 68°C for 1 min and 30 sec. And then the T7 promoter and terminator primers were added into the mixture to amplify the full-length gene, the PCR was performed at 95°C for 2 min, with 30 cycles of 95°C for 30 sec, 50°C for 30 sec and 68°C for 1 min and 30 sec, and a final extension of 68°C for 5 min.

The resulting full-length PCR product was verified for correct size on an agarose gel, cut from the gel and purified using QIAquick Gel Extraction Kit (Qiagen). The purified PCR fragment was cloned to pBS(KS+) vector after double digestion of both vector and insert with *Xba*I and *Xho*I. The ligation products were transformed into *E. coli* MB3436 and selected on LB ampicillin IPTG/ X-gal medium. Several white colonies were picked for plasmid isolation and restriction analysis, and the hybrid gene from one recombinant was sequenced. The overnight culture of this clone was tested for its ability to degrade cyanide.

The construction of the other three hybrid proteins were essentially as described above, except the primers used for each were different (Table 8): p3736 with primers C1-R2 and OxB1-F2; p3864 with primers OxB1-R3 and C1-F3; p3866 with primers OxB-F4 and C1-R4. The full-length hybrid gene of p3736 and p3866 were digested with

*Hind*III and *Xho*I and cloned to the same sites of p3504 because there was an *Xba*I restriction site located at nt 831 of the OxB-1 nitrilase gene.

Results

Cyanide-degrading activity of *Bacillus* sp. OxB-1 nitrilase

The synthetic OxB-1 nitrilase gene with optimal *E.coli* codon usage was previously constructed in the lab. A frameshift mutation existed on the synthetic gene and was repaired to get the wild type gene for OxB-1 nitrilase. The OxB-1 gene was cloned into the expression vector pET26b and induced to express the protein. The cell culture was lysed by sonication. Different concentrations of cyanide was added to the cell lysate to test the cyanide-degrading activity but no cyanide degrading activity was detected.

Western blot

To confirm the expression of the OxB-1 nitrilase, western blotting was performed using the cell lysate samples from the induced cell culture. A polyclonal antibody against the cyanide hydratase and dihydratase was used to detect the nitrilase protein. Cyanide dihydratase of *pumilus* C1 was used as positive control. From the western blot (Fig. 6), the expression of the OxB-1 nitrilase was confirmed.

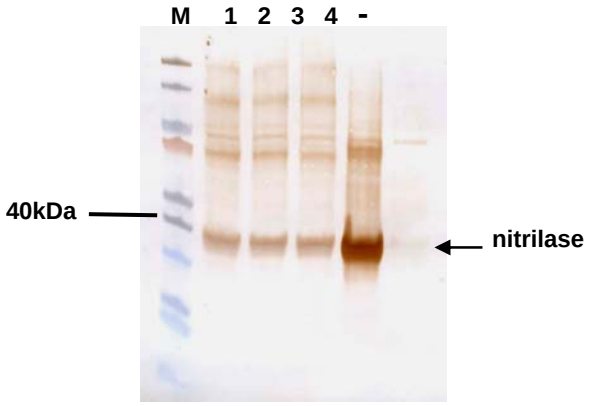

FIG. 6. Western blot of the OxB-1 nitrilase and CynD of *B. pumilus* C1.
Lane 1-3: OxB-1; 4: pBS-*B. pumilus* C1; - : pBS (KS+) control

Cyanide degrading activity of OxB-1Δ(192-204)

From the protein alignment of the OxB-1 nitrilase with the cyanide dihydratases of *Bacillus pumilus* C1 and P. *stutzeri* AK61 (Fig. 5), there is a 10 amino acid insertion (192-204) in OxB-1 nitrilase that CynD does not have. Site-directed mutagenesis was performed to delete these 13 amino acids. Then the protein was expressed and the activity of OxB-1Δ(192-204) was tested. There was again no detectable cyanide-degrading activity.

Cyanide degrading activity of the hybrid proteins

In order to roughly locate the amino acids differences that might be responsible for the substrate specificity, hybrid proteins with regions from the nitrilise of OxB-1 and CynD of *B. pumilus* C1 were constructed (Fig. 7). First, two separate PCR reactions were performed to get the DNA fragments of OxB-1 nitrilase and $CynD_{pum}$ separately. These PCR fragments were combined as the DNA template to get the full-length gene of the hybrid protein. From the sequences alignment (Fig. 5), there are many amino acids differences with charge changes located in the downstream region of these two proteins

at the site near the deletion (192-204). The site to construct the first set of hybrid proteins (p3730 and p3736) was chosen 15 amino acids downstream of the deletion (192-204) of OxB-1 nitrilase. The site to construct the second set of hybrid proteins (p3864 and p3866) was selected about 50 amino acids upstream of the deletion (192-204) of OxB-1 nitrilase. No cyanide degrading activity was detected for any the four hybrid proteins constructed. Therefore, I decided to not continue this project.

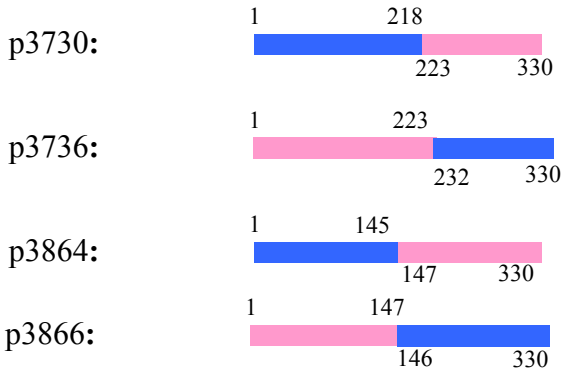

FIG. 7. Site of the creation of the hybrid proteins.
: amino acids from OxBΔ1
: amino acids from $CynD_{pum}$

Discussion

The OxB-1 nitrilase of *Bacillus* sp. is similar at the amino acid level with the cyanide dihydratases from *B. pumilus* C1 and *P. stutzeri* AK61. These enzymes are all nitrilases but they recognize different substrates. I used this difference between them as a model to study the substrate specificity and recognition of nitrilases.

First, I constructed OxB-1Δ(192-204) to delete 13 amino acids that the cyanide dihydratases do not have. I suspected that these 13 amino acids may relate to different substrate specificities. Then, I constructed four hybrid proteins made from OxB-1 nitrilase and CynD of *B. pumilus* C1. The site used to construct the first set of hybrid proteins was chosen because the downstream regions of these two proteins are divergent with many charge changes. When the first two hybrid proteins failed to degrade cyanide, I thought the reason may be that the site (amino acid 218) I choose is too close to the deletion (192-204) of OxB-1 nitrilase and the hybrid proteins could not fold correctly. A second set of hybrid proteins was constructed with the hybrid site located upstream of the deletion (192-204) of OxB-1 nitrilase. The second set of hybrid proteins was still unable to degrade cyanide. The location of the hybrid junction of these hybrids all avoided the region of the Glu-Lys-Cys catalytic triad. However, there may be other complicating factors in the constructed hybrid proteins that affect protein folding or abolish interactions around the Glu-Lys-Cys catalytic triad (Glu48- Lys130- Cys163).

To better identify the amino acids responsible for substrate specificity along the entire coding gene, I analyzed the alignment of the amino acids of OxB-1 nitrilase with cyanide dihydratases from *B. pumilus* C1 and *P. stutzeri* AK61. Those most conserved residues in the two cyanide-degrading enzymes that differ in OxB-1 may contribute to substrate recognition. Therefore, the positions of OxB-1 nitrilase could be mutated through site-directed mutagenesis to those residues that occur most frequently in the multiple sequence alignment (labeled with arrows in Fig. 8). The relation between these

residues and the catalytic triad (Glu48- Lys130- Cys163, labeled in box in Fig. 8) was also taken into consideration. The non-conserved C-terminus was ignored.

There are many factors that contribute to the substrate specificity of the nitrilases. The selected mutagenesis sites of OxB-1 nitrilase may not make this enzyme degrade cyanide either. There may be some other sites that I did not suspect that convert the substrate specificity. Random mutagenesis is another strategy that could be employed. The error-prone PCR may be performed on the gene of OxB-1 nitrilase and the generated library of mutants could be screened to test the ability of cyanide-degrading. If some mutants with cyanide-degrading activity are found, the substrate specificity study will be performed on these mutants by sequence analysis and structure study.

At the end of this project, I expect that the substrate specificity of OxB-1 nitrilase would be converted to degrade cyanide and the amino acids or domains responsible for substrate specificity will be identified. However, this has not yet been achieved.

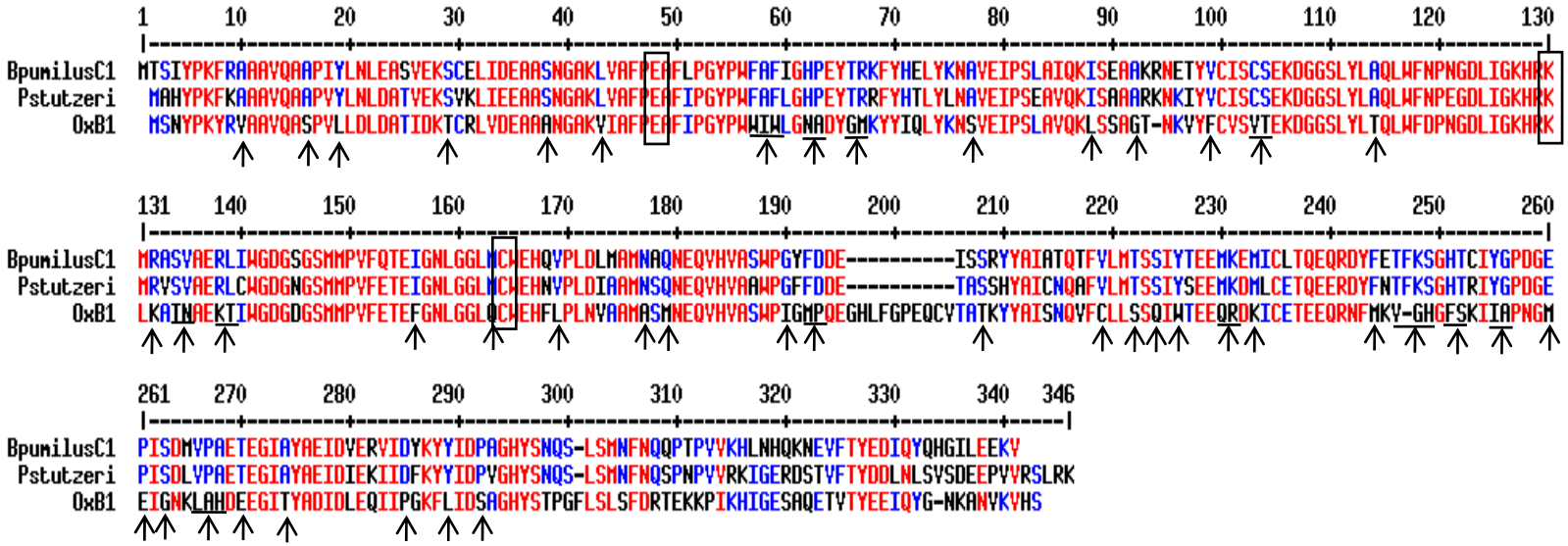

FIG. 8. Target residues of the site-directed mutagenesis of the OxB-1 nitrilase.
The aa in red color are similar in all 3, in blue are similar for 2 of 3 sequences, and in black are not conserved.
The residues labeled with arrows are potential targets residues of the mutagenesis.
The resides labeled in box are the conserved catalytic triad (Glu-Lys-Cys). The non-conserved C-terminus was ignored

CHAPTER V

SUMMARY AND CONCLUSIONS

There is a potential demand for cyanide-degrading enzymes with better properties that can tolerate the adverse conditions of cyanide waste bioremediation in industry. The existing cyanide-degrading nitrilases all have their limits for use as agents in bioremediation under different conditions, and the crystal structures of the microbial nitrilases including cyanide dihydratase still remain unknown. Therefore, more work needs be done to engineer the cyanide-degrading enzymes for better properties in industrial cyanide remediation. The study of these mutants will provide insight into the underlying mechanisms of catalysis and the knowledge of the protein structure and function.

Directed evolution is a powerful tool to improve the properties of proteins. The approach has the advantage that detailed knowledge about the relationship between molecular structure and function is not required. Random mutagenesis was employed to construct a library of $CynD_{pum}$ mutants in this study. Different error-prone PCR conditions were used to get the optimal mutation frequency. The PCR condition was optimized at 130ng of target DNA template in a 50 μl reaction, 0.2 mM of $MnCl_2$, and 20 cycles of amplification. The screening system for improved mutants is based on a micro-plate assay that changes in color (red→yellow) upon hydrolysis of cyanide. We developed a high throughput two-dimensional screening strategy that was carried out at two different pH values.

Two $CynD_{pum}$ mutants (mutant C5 and H7) with higher pH tolerance were generated by random mutagenesis and selection in this study. Mutants C5 and H7 can degrade cyanide at pH10 while the wild-type $CynD_{pum}$ can not. Both mutants have three amino acids changes from the wild-type enzyme. Alleles were constructed to study the effect of each single amino acid change on the improved activity, and the cyanide-degrading activities of these mutants were tested. The pH activity profiles and thermal stability of purified proteins were measured. The pH profile of mutant C5 may suggest that the pH tolerance is not due to higher catalytic activity but due to other factors such as a structural effect. The thermal stability of mutant C5 was greatly enhanced at 42^{o}C. It maintained about twice the activity compared to the wild-type enzyme after 8 and 48 hour incubation. Q86R, E96G, and D254E may act together to increase the stabilization of the spiral quaternary structure at higher pH or temperature. Whereas E327G might be one key amino acid that contributes to the stabilization of the spiral structure at higher pH. E327G is located in the C-terminal extension which has been implicated in stabilizing the oligomer form.

Overall, this work demonstrated the successful improvement of a cyanide dihydratase by increasing activity at alkaline pH through random mutagenesis for the first time. We were able to increase the activity of the mutated enzymes over a broader pH range. None of the amino acid mutations found in mutant C5 or H7 have been previously described to have an influence on the pH activity profile or thermal stability.

The goal of the second project was to convert the substrate specificity of OxB-1 nitrilase to degrade cyanide and identify the amino acids or domains responsible for

substrate specificity. The OxB-1 nitrilase of *Bacillus* sp. is similar at the amino acid level with the cyanide dihydratases from *B. pumilus* C1 and *P. stutzeri* AK61. These enzymes are all nitrilases but they recognize different substrates. Their overall similarity provides a good model to study the substrate specificity of nitrilase. One deletion mutant and four hybrid proteins were constructed based on the alignment information. The constructed proteins were all unable to degrade cyanide.

REFERENCES

1. **Aharoni, A., L. Gaidukov, O. Khersonsky, S. M. Gould, C. Roodveldt, and D. S. Tawfik.** 2005. The "evolvability" of promiscuous protein functions. Nat. Genet. 37:73-76.

2. **Aguinaldo, A. M. and F. H. Arnold.** 2003. Staggered extension process (StEP) in vitro recombination. Methods Mol. Biol. 231:105-110.

3. **Akcil, A. and T. Mudder.** 2003. Microbial destruction of cyanide wastes in gold mining: process review. Biotechnol Lett. 25:445-450.

4. **Alsh, D. W.** 2005. Cyanide: a ubiquitous product of combustion in modern fires. FireEMS. May:6-11.

5. **Arnold, F. H.** 1998. Design by directed evolution. Acc. Chem. Res. 31:125-13

6. **Benerjee, A., R. Sharma, and U. C. Banerjee.** 2002. The nitrile-degrading enzymes: current status and future prospects. Applied Micro. Biotech. 60:33-44.

7. **Bloom, J. D., M. M. Meyer, P. Meinhold, C. R. Otey, D. MacMillan, and F. H. Arnold.** 2005. Evolving strategies for enzyme engineering. Curr. Opin. Struc. Bio. 15:447-452.

8. **Brenner, C.** 2002. Catalysis in the nitrilase superfamily. Curr. Opin. Struct. Biol. 12:775-782.

9. **Chen, R.** 2001. Enzyme engineering: rational design versus directed evolution. Trends Biotechnol. 19:13-14.

10. **Cherry, J. R. and A. L. Fidantsef.** 2003. Directed evolution of industrial enzymes: an update. Curr. Opin. Biotechnol. 14:438-443.

11. **Cirino, P. C., K. M. Mayer, and D. Umeno.** 2003. Generating mutant libraries using error-prone PCR. Methods Mol. Biol. 231:3-9.

12. **Coco, W. M.** 2003. RACHITT: Gene family shuffling by random chimeragenesis on transient templates. Methods Mol. Biol. 231:111-127.

13. **Ebbs, S.** 2004. Biological degradation of cyanide compounds. Environ. Biotechnol. 15:231-236.

14. **Farinas, E. T., T. Bulter, and F. H. Arnold.** 2001. Directed enzyme evolution. Curr. Opin. Biotechnol. 12:545-551.

15. **Figueira, M. M, V. S. T. Viminellia, and V. R. Linardi.** 1995. Bacterial degradation of metal cyanide complexes, p. 333-339. Biohydrometallurgical processing, University of Chile, Santiago.

16. **Ingvorsen, K., B. Hojer-Pedersen, and S. E. Godtferdsen.** 1991. Novel cyanide hydrolyzing enzyme from *Alcaligenes xylosoxidans* subsp. *Denitrificans*. Appl. Environ. Microbiol. 57:1783-1789.

17. **Jandhyala, D., M. Berman, P. R. Meyers, B. T. Sewell, R. C. Willson, and M. J. Benedik.** 2003. CynD, the cyanide dihydratase from *Bacillus pumilus*: Gene cloning and structural studies. Appl. Environ. Microbiol. 69:4794-4805.

18. **Jandhyala, D. M., R. C. Willson, B. T. Sewell, and M. J. Benedik.** 2005. Comparison of cyanide-degrading nitrilases. Appl. Micro. Biotechnol. 68:327-335.

19. **Jenks, W. R.** 1985. Cyanide: hydrogen cyanide, p. 334. *In* Kirk-Othmer concise encyclopedia of chemical technology, John Wiley & Sons, Somerset, NJ.

20. **Kato, Y., K. Nakamura, H. Sakiyama, S. G. Mayhew, and Y. Asano.** 2000. Novel heme-containing lyase, phenylacetaldoxim dihydratase from *Bacillus* sp. strain OxB-1: purification, characterization, and molecular cloning of the gene. Biochemistry 39:800-809.

21. **Meyers, P. R., O. Gokool, D. E. Rawlings, and D. R. Wood.** 1991. An efficient cyanide-degrading *Bacillus pumilus* strain. J. Gen. Microbiol. 137:1397-1400.

22. **Meyers, P. R., D. E. Rawlings, D. R. Wood, and G. G. Lindsey.** 1993. Isolation and characterization of a cyanide dihydratase from *Bacillus pumilus* C1. J. Bacteriology 175: 105-6112

23. **Miyazaki, K. and M. Takenouchi.** 2002. Creating random mutagenesis libraries using megaprimer PCR of whole plasmid. BioTechniques 33:1033-1038.

24. **Murakami, H., T. Hohsaka, and M. Sisido.** 2003. Random insertion and deletion mutagenesis. Methods Mol. Biol. 231:53-64.

25. **Neylon, C.** 2004. Chemical and biochemical strategies for the randomization of protein encoding DNA sequences: library construction methods for derected evolution. Nucleic Acids Res. 32: 1448-1459.

26. **Ostermeier, M. and S. Lutz.** 2003. The creation of ITCHY hybrid protein libraries. Methods Mol. Biol. 231:129-141.

27. **O'Reilly, C. and P. D. Turner.** 2003. The nitrilase family of CN hydrolyzing

enzymes- a comparative study. Appl. Microbiol. 95:1161-1174.

28. **Pace, H. and C. Brenner.** 2001. The nitrilase superfamily: classification, structure and function. Genome Biology 2: 001.1-0001.9.

29. **Stemmer, W. P. C.** 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370:389-391

30. **Stevenson, J., M. Botz, T. Mudder, A. Wilder, R. Richins, and B. Brudett.** 1995. Cyanisorb recovers cyanide. Min. Env. Mange. 3:9.

31. **Sewell, B. T., M. N. Berman, P. R. Meyers, D. Jandhyala, and M. J. Benedik.** 2003. The cyanide degrading nitrilase from *Pseudomonas stutzeri* AK61 is a two-fold symmetric, 14-subunit spiral. Structure 11:1-20.

32. **Sewell, B. T., R. N. Thuku, X. Zhang, and M. J. Benedik.** 2005. The oligomeric structure of nitrilases: the effect of mutating interfacial residues on activity. Annals of New York Academy of Sciences 1056:153-159.

33. **Topcu, Z.** 2000. An optimized recipe for cloning of the polymerase chain reaction-amplified DNA inserts into plasmid vectors. Acta. Biochim. Pol. 47:841-846.

34. **United States Department of Health and Human Services.** September 2004. Toxicological profile for cyanide, Agency for Toxic Substances and Diseases Registry, Washington, DC.

35. **Wahler, D. and J. L. Reymond.** 2001. High-throughput screening for biocatalysts. Curr. Opin. Biotechnol. 12:535-544.

36. **Wang, P., D. E. Mathews, and H. D. VanEtten.** 1992. Purification and characterization of cyanide hydratase from the phytopathogenic fungus *Gloeocercospora sorghi*. Archives of Biochemistry and Biophysics 298:569-575.

37. **Wang, L., J. Xie, and P. G. Schultz.** 2006. Expanding the genetic code. Annu. Rev. Biophys. Biomol. Struct. 35:225-49.

38. **Wang, T. W., H. Zhu, X. Y. Ma, T. Zhang, Y. S. Ma, and D. Z. Wei.** 2006. Mutant library construction in directed molecular evolution. Mol. Biotechnol. 34:55-68.

39. **Watanabe, A., K. Yano, K. Ikebukuro, and I. Karube.** 1998. Cloning and expression of a gene encoding cyanide from *Pseudomonas stutzeri* AK61. Appl. Micro. Biotechnol. 50:95-97.

40. **Way, J. L.** 1981. Pharmacologic aspects of cyanide and its antagonism, p. 29-49. *In* Cyanide in biology, Academic Press, London, UK.

41. **Wong, T. S., K. L. Tee, B. Hauer, and U. Schwaneber.** 2004. Sequence saturation mutagenesis (SeSaM): a novel method for directed evolution. Nucleic Acids Res. 32:e26-34.

42. **Woodward, J. D., B. W. Weber, M. P. Scheffer, M. J. Benedik, A. Hoenger, and B. T. Sewell.** 2007. Helical structure of unidirectionally shadowed metal replicas of cyanide hydratase from *Gloeocercospora sorghi*. J. Structural Biology in press doi:10.1016/j.jsb.2007.09.019

43. **Young, C. A. and T. S. Jordan.** 1995. Cyanide remediation: Current and past technologies, p. 104-128. Proceedings 10th Annual Conference on Hazard Waste Research, Manhattan, KS.

44. **Yen, D., J. Tsai, L. Wang, W. Kao, S. Hu, C. Lee, and J. Deng.** 1995. The clinical experience of acute CN^- poisoning. American J. Emergency Medicine. 13:524-528.

45. **Zaccolo, M., D. M. Williams, D. M. Brown, and E. Gheradi.** 1996. An approach to random mutagenesis of DNA using mixtures of triphosphate derivatives of nucleoside analogues. J Mol Biol. 255:589-603.

VITA

Name: Lan Wang

Address: Department of Biology, BSBE 307
Texas A&M University 3258
College Station, TX, 77843

Email Address: lanelel@tamu.edu

Education: B.S., Biology, Beijing Normal University, 2000
M.S., Microbial and Biochemical Pharmaceutics, Institute of Medicinal Biotechnology, Peking Union Medical College, 2003
M.S., Microbiology, Texas A&M University, 2008