LINKAGE OF A NITRILASE-CONTAINING Nit1C GENE CLUSTER TO CYANIDE UTILIZATION IN *Pseudomonas fluorescens* NCIMB 11764

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Pseudomonas fluorescens NCIMB 11764 (Pf11764) is uniquely able to grow on the poison cyanide as its sole nitrogen source. It does so by converting cyanide oxidatively to carbon dioxide and ammonia, the latter being assimilated into cellular molecules. This requires a complex enzymatic machinery that includes nitrilase and oxygenase enzymes the nature of which are not well understood. In the course of a proteomics analysis aimed at achieving a better understanding of the proteins that may be required for cyanide degradation by Pf11764, an unknown protein of 17.8 kDa was detected in cells exposed to cyanide. Analysis of this protein by ESI-coupled mass spectrometry and bioinformatics searches gave evidence of strong homology with a protein (Hyp1) of unknown function (hypothetical) present in the bacterium *Photorhabdus luminescens subsp. laumondii TTO1* (locus plu_1232). A search of available microbial genomes revealed a number of Hyp1 orthologs the genes of which are found in a conserved gene cluster known as Nit1C. Independent studies revealed that in addition to Hyp1, Pf11764 possesses a gene (*nit*) specifying a nitrilase enzyme whose closest homologue is a nitrilase found in Nit1C gene clusters (77% amino acid identity). DNA sequence analysis has further revealed that indeed, $hyp1_{Pf11764}$ and $nit_{Pf11764}$ are contained in a cluster that includes also a gene specifying an oxygenase. Given the possible connection of Nit1C-endoded nitrilase and oxygenase enzymes to enzymatic cyanide degradation, there is strong reason for thinking that the genes specifying these enzymes contribute to bacterial growth on cyanide in those bacteria containing the Nit1C cluster. Because the biological function of the Hyp1 protein is currently unknown, it was cloned and the protein expressed in *E. coli* so that its properties could further be

explored. Unfortunately, the expression of the protein in an insoluble form complicated these analyses. However, at least two lines of evidence suggest a possible role as a regulator of gene expression. First, over-expression of the protein was accompanied by the parallel elevation of the putative vector-encoded β-lactamase, implying that $Hyp1_{Pfl1764}$ can affect the expression of other genes. Second, a comparison of the amino acid sequence of select peptide fragments of $Hyp1_{Pfl1764}$, by conducting searches for homology with proteins in the existing nonredundant protein database, consistently revealed motifs in common with those present in bacterial response regulators that are part of two-component signal transduction systems widely distributed in bacteria.

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LIST OF ABBREVIATIONS

CNO	Cyanide oxygenase
EC	Enzyme commission
ESI-MS	Electrospray ionization mass spectrometry
h	Hour
KCN	Potassium cyanide
kDa	Kilodalton
min	Minute
MS	Mass spectrometry
Mol wt	Molecular weight
Na-K buffer	Sodium potassium buffer
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
Pf	*Pseudomonas fluorescens*
PVDF	Polyvinylidine difluoride
SDS	Sodium dodecyl sulfate

CHAPTER I

INTRODUCTION

Physicochemical Properties of Cyanide and Its Occurrence

Cyanide exists commonly as hydrogen cyanide (HCN/CN^-, CAS No. 74-90-8) or it salts - sodium cyanide (NaCN, CAS No. 143-33-9) and potassium cyanide (KCN, CAS No. 151-50-8). Hydrogen cyanide (free cyanide) is a weak acid (pKa 9.3) in aqueous solution and boils at room temperature (25^0C) (Fuller, 1988). At physiological pH (7.0) therefore it, exists predominantly in the protonated form and equilibrates between dissolved and gaseous species. Cyanide is a strong nucleophile and once having entered biological tissue it readily binds to various molecules, most notably, iron in heme containing cytochrome oxidase. This leads to inhibition of respiration which accounts in large part for cyanide toxicity (Knowles, 1988). In addition to cytocrome oxidase it also inhibits various metalloenzymes (Solomonson, 1981).

Cyanide arises in the environment by natural and anthropogenic means. Large quantities are employed in various industrial processes such as metal plating, steel tempering and mining (Agency for Toxic Substances and Disease Registry, 1993; Homan, 1988). These represent important sources of potential cyanide pollution in the environment. A number of organisms produce cyanide as a natural product including various plants (e.g., maize, millet, sorghum and sweet potatoes) as well as certain bacteria, fungi, and algae (Conn, 1980; Castric, 1981; Knowles and Bunch, 1986; Poulton, 1988). While the role of cyanide production (cyanogenesis) is not entirely clear it is generally believed to play a role in defense against competitors (Blumer and Haas, 2000).

Cyanide Tolerance

Increasing tolerance to cyanide as a respiratory inhibitor is generally acquired by the elaboration of cyanide-resistant cytochrome oxidases (Cunningham and Williams, 1995; Rhoads et al, 1998). In response to cyanide, for example, *Pseudomonas aeruginosa* and *Bacillus cereus* elaborate cytochrome oxidases capable of tolerating inhibitor concentrations as high as 1 mM (Knowles and Bunch, 1986). Breakdown of cyanide by various detoxifying enzymes is believed also to confer increased resistance. One of the main enzymes in animals for detoxifying cyanide is rhodanese (thiosulfate cyanide sulfurtransferase) (E C 2.8.1.1). Rhodanese catalyses the conversion of cyanide to less toxic thiocyanate as shown in equation 1 (Westley, 1981). In plants the enzyme, β-cyanoalanine synthase (E C 4.4.1.9) is thought to be an important cyanide

$$S_2O_3^{-2} + HCN \xrightarrow{\text{rhodanese}} HSCN + SO3^{-2} \qquad (1)$$

detoxifying enzyme. It catalyzes the condensation of cyanide and cysteine as shown in equation 2 (Castric, 1981). In fungi, particularly phytopathogenic fungi (*Stemphylium loti,*

$$HCN + HSCH_2-\underset{NH_2}{\overset{COOH}{C}}-H \xrightarrow{\beta\text{-cyanoalanine synthase}} \underset{\beta\text{-cyanoalanine}}{COOH-\overset{NH_2}{CH}-CH_2-CN} + H_2S \qquad (2)$$

Gloecercospora sorghi) (Fry and Millar, 1972; Wang et al, 1992), cyanide is thought to be detoxified by cyanide hydratase (E.C.4.2.1.66) which converts cyanide to less toxic formamide (equation 3). Bacteria produce a related enzyme, cyanide dihydratase (CynD) (also known as cyanide specific nitrilase or cyanidase) (E.C. 3.5.5.1) which converts cyanide to formate and

$$HCN + H_2O \xrightarrow{\text{cyanide hydratase}} HCONH_2 \qquad (3)$$

ammonia (equation 4). Although the role of this enzyme is believed to be one of detoxification,

$$HCN + 2H_2O \xrightarrow{\text{Cyanide dihydratase}} HCO_2H + NH_3 \qquad (4)$$

because of the high concentrations of cyanide required for activity (1.5-10 mM) (Jandhyala et al., 2005) it is uncertain whether this is its true role. The occurrence of CynD enzymes has thus far been described for *Bacillus pumilus* (Meyers et al., 1993) *Pseudomonas stutzeri* (Jandhyala et al., 2003), and *Alcaligenes xylosoxidans* (Ingvorsen et al., 1991). Recent studies in our laboratory led to the discovery of a related enzyme in the cyanide-utilizing bacterium, *P. fluorescens* (strain NCIMB 11764) (Fernandez and Kunz, 2005). Because this enzyme was shown to be part of a complex of enzymes required for cyanide oxidation, strong interest in characterizing it and other enzymes of the complex including also NADH oxidase (Nox), NADH peroxidase (Npx) and carbonic anhydrase (CA), was raised. Further studies in our laboratory suggesting a role of the Pf11764 CynD enzyme in conferring cyanide utilization represent the first of its kind in bacteria.

Nutritional Utilization of Cyanide as a Growth Substrate

The isolation of bacteria able to use cyanide for growth has been reported in a number of instances (Adje et al., 1999; Finnegan et al., 1991; Fry and Miller, 1972; Harris and Knowles, 1983a; Silva-Avalos et al., 1990; Skrowronski et al., 1969; Ware and Painter, 1955), however, the biochemical basis of this ability has remained generally unknown. Bacteria have been reported to use cyanide as a sole nitrogen source but there are no reports of it serving as the sole source of carbon and energy. It has been suggested that this is probably because at the concentrations needed for this purpose it is too toxic (Knowles and Bunch, 1986; Kunz et al., 1992; Raybuck, 1992). Only in the case of Pf11764 has the enzymatic basis of cyanide utilization been determined to any significant extent (Kunz et al., 1994; Kunz et al., 2001;

Fernandez et al., 2004; Fernandez, 2004; Fernandez and Kunz, 2005). In this case cyanide is known to be oxidized to carbon dioxide and ammonia, the latter then being assimilated into cellular nitrogen-containing molecules. Two principal enzymatic processes appear to be involved, one in which molecular oxygen is inserted into the substrate to give formate (and ammonia) and the second in which formate is further oxidized to carbon dioxide. The first of these processes involves the complex of enzymes referred to earlier including a putative CynD enzyme, Nox, Npx and CA enzymes (collectively referred to as the CNO complex) (Fernandez et al., 2004; Fernandez and Kunz, 2005). CNO consumes two equivalents of NADH and one of O_2 when cyanide is cleaved (equation 5). The oxidation of formate then is further catalyzed by an NAD^+-linked formate dehydrogenase (equation 6). Both activities are simultaneously induced when cells are grown on cyanide (KCN) or otherwise exposed to it under minimal growth conditions. A perplexing question concerning the enzymes and proteins that are part of

$$HCN + O_2 + 2NADH + H^+ \xrightarrow{CNO} HCO_2H + NH_3 + 2NAD^+$$

$$HCO_2H + NAD^+ \xrightarrow{FDH} CO_2 + NAD^+ \qquad (6)$$

the of the machinery for cyanide oxidation pertains to the findings reported by Harris and Knowles (1983b) (who initially isolated Pf11764 and first studied the basis of cell-free cyanide conversion), that a polypeptide of ~17 kDa was present in cell-extracts of bacteria grown on cyanide but absent from cells not grown on cyanide. These investigators first hypothesized that this protein was needed for enzymatic conversion but its involvement was not further investigated. In order to determine what the involvement of this protein in cyanide metabolism might be experiments were carried out to ascertain whether its formation was at all coordinated with induction of CNO and FDH enzymes shown previously to be required for cyanide

breakdown. These experiments showed that indeed a protein of ~17 kDa polypeptide could also be detected in cell extracts as first reported by Harris and Knowles (1983b). What follows in this thesis is an account of experiments on the characterization of this protein.

CHAPTER II

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Cultivation Conditions

All bacterial strains and plasmid used in this study are listed in Table 1. *Pseudomonas fluorescens* NCIMB 11764 (Pf11764) cells were routinely subcultured from a frozen stock (-80^{0}C) on Lennox agar plates (Lennox, 1955) at 30^{0}C the components of which have been described previously (Wang, 1995). The minimal medium employed for Pf11764 cultivation consisted of 67 mM KH_2PO_4 (pH 7.0), 1.6 μM $MgSO_4.7H_2O$, 6 μM $FeSO_4$. $7H_2O$, 10 mM glucose as a carbon source and 1 mM ammonium chloride as a nitrogen source. The upregulation of proteins in response to cyanide was accompanied by inoculating a single colony to 100 ml minimal medium. This culture was grown for 48 hours at 30°C on a shaker (200 rpm) at which time the entire culture transferred (10% inoculum) to a flask containing 1 liter of the same medium. This culture was grown for 24 hour at which time 0.1 mM KCN was added. After 3 hours, cells were harvested and washed in 50 mM Na_2HPO_4-KH_2PO_4 (pH 7.0) (Na-K) buffer before being stored at -80°C.

Escherichia coli cells were routinely cultured at 37^{0}C in Luria-Bertini (LB) medium (Sambrook and Russel, 2001), supplemented with kanamycin and/or ampicillin at a final concentration of 50 μg/ml and 100 μg/ml respectively if appropriate.

Table 1: Strains and plasmids used in this study.

Strains and plasmids	Genotype or Characteristics	Source or Reference
Strains		
P. fluorescens NCIMB11764	Wild type, Pf11764	Dr. Daniel A. Kunz
Escherichia coli DH5α-T1^{R}	(Cloning host) F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A	Invitrogen, CA, USA
Escherichia coli TOP10	(Cloning host) F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G	Invitrogen, CA, USA
Escherichia coli BL21 Star^{TM} (DE3)	(Expression host) F– *omp*T *hsd*SB(rB–, mB–) *gal dcm rne*131 (DE3)	Invitrogen, CA, USA
DK1290	*Escherichia coli* BL21 Star^{TM} (DE3) containing pGK1 plasmid	This study
Plasmids/Cloning Vectors		
pCR4®-TOPO	Lac promoter, Kanamycin and Ampicillin resistance, 3956 bp	Invitrogen, CA, USA
pET101/D-TOPO®	T7 promoter, Ampicillin resistance, V5 epitope, 5753 bp	Invitrogen, CA, USA
pGK1	pET101/D-TOPO® plasmid containing *hyp1*gene	This study

Preparation of Cellular Extracts

Frozen cells of Pf11764 were suspended in twice the volume (2 ml of buffer per gram of cells) of chilled 50 mM Na_2HPO_4-KH_2PO_4 (pH 7.0) buffer containing 50 μg/ml DNase and broken in a chilled French press at 20,000 psi. Following cell breakage the preparation was held at room temperature for 5 minutes to allow time for DNA digestion and the preparation was centrifuged at 30,000 x g (30 min at 4°C). The supernatant (crude extract) was retained and again centrifuged at 150,000 x g for 90 min at 4 °C with the supernatant (cytosolic cell extract) recovered and frozen at -80°C until use.

Protein Determination

The protein content of cell extracts was determined using the Lowry method (Lowry et al., 1951). The standard procedure involved adding 5 μl of protein sample to 0.2 ml of water to which was added reagent A (prepared fresh by mixing 0.5% $CuSO_4 \cdot 5H_2O$ made in 1% sodium tartrate with 2% Na_2CO_3 prepared in 0.1 N NaOH in 1:50 ratio). After 10 minutes, 0.1 ml of 1 N Folin-phenol reagent (2N) was added and the absorbance read at 750 nm after 30 minutes. The sample protein concentration was determined using a standard curve obtained with bovine serum albumin as a standard.

Discontinuous SDS-PAGE of Proteins

16% SDS-PAGE (Laemmli, 1970) gels with a 5% stacking gel were used regularly for protein analysis. The 16% acrylamide separating gel was prepared by mixing 5.35 ml of acrylamide stock solution (30% w/v acrylamide and 0.8% w/v N'N'-bis-methylene-acrylamide in ddH2O), 2.5 ml of 1.5 M Trizma (pH 8.8), 100 μl of 10% w/v SDS, 2.05 ml ddH2O, 50 μl ammonium persulfate (10 % w/v) and 5 μl of N,N,N',N'-tetramethylene ethylenediamine (TEMED) was added. A vertical gel (8.4 x 5.3 cm) was cast leaving a 2 cm space that was

layered with isopropyl alcohol and the gel was allowed to polymerize at room temperature for 45 minutes. The isopropyl alcohol layer was washed with ddH_2O and a 5% stacking gel prepared by mixing 0.65 ml of acrylamide stock solution, 1.25 ml of 0.5 M Trizma (pH 6.8), 50 µl of 10% w/v SDS, 3.05 ml of ddH_2O, 25 µl of ammonium sulfate (10 % w/v) and 5 µl of TEMED. The gel was allowed to polymerize for 45 min and placed in an electrophoresis tank containing running buffer (25 mM Trizma, 192 mM glycine and 0.1% w/v SDS, pH 8.3). The samples were denatured by heating at 95°C for 5 min after being mixed with sample buffer (150 mM dithiothreitol, 150 mM Trizma pH 6.8, 21% w/v glycerol, 6% w/v lithium dodecyl sulfate and 0.003% w/v bromophenol blue) in a ratio of 2:1. Samples were cooled, loaded onto the gel, and electrophoresis performed at 4 °C for 55 min at a constant voltage of 200 V. The gels were stained with Coomassie Blue staining solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid, 0.1% (w/v) Coomassie Brilliant Blue R-250 in ddH_2O) for 30 min and destained with 40% (v/v) methanol and 10% (v/v) glacial acetic acid in ddH_2O overnight. The molecular masses of protein markers were: phosphorylase B, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

Electroblotting

Cyanide induced cytosolic protein fraction was transferred onto a polyvinyldene difluoride (PVDF) (Bio-Rad) membrane followed by SDS-PAGE for amino acid sequencing by electroblotting. PVDF membranes obtained from Bio-Rad were placed in 100 % methanol for a few seconds, washed with water and soaked in transfer buffer (25 mM Trizma, 192 mM glycine and 20% v/v methanol, pH 8.3) for half an hour. Following SDS-PAGE, gels were soaked in transfer buffer for no more than 15 min. A sandwich consisting of filter pad, blotting paper, gel, PVDF membrane, blotting paper and filter pad in that order were layered (while soaking in

transfer buffer) from the negative to the positive electrode of the Bio-Rad transfer cell. The cell was placed in the blotting chamber, filled with the transfer buffer and blotted overnight at 4 °C and 30 V. After the transfer, membranes were washed three times (5 min) with distilled water, and then stained (0.025 % commasie blue R-250 in 40% MeOH) and destained (50 % methanol) for 5 min and 15 min, respectively. Bands of interest were excised using dust free scissors after the membranes were allowed to dry at room temperature.

N-terminal Amino Acid Analysis

The N-terminal sequence of the Hyp1 protein was fetermined after eletroblotting the sepatated protein bands drom an SDS-gel onto a PVDF membrane using established methods (Fernandez and Kunz, 2005). Sequence analysis was performed at the Protein Chemistry Laboratory of the Texas A&M University at College Station, USA.

Two Dimensional Gel Electrophoresis (O'Farrell, 1975)

Sample Cleanup for 2D Gels

Impurities in cell extracts (detergents, salts etc) were removed and pelleted by using a ReadyPrep 2-D Cleanup Kit from Bio-Rad (Hercules, CA, USA). The recovered protein pellet fraction was washed with sterile ddH_2O, air dried and was resuspended very thoroughly by mixing with 200 µl of 2-D rehydration/sample buffer from a Bio-Rad Ready Prep Sequential Extraction Kit containing 5 M urea, 3 M thiourea, 2% CHAPS, 2% SP310, 40 mM Tris, 0.2% Bio-lyte (w/v), 50 mM DTT and trace amount of Bromophenol Blue (BPB).

First Dimension Isoelectric Focusing

Protein (300 µg) was added to a 11 cm pH 4-7 immobilized pH Gradient strip (IPG) by rehydration overnight in an 185 ul volume of rehydration/sample buffer. The IPG strips were covered with mineral oil to avoid evaporation of the sample. Iso-electric focusing was performed

by using a Protean IEF cell form Bio-Rad (Biorad, Hercules, CA, USA), in four stages. A 250 V for 20 min was followed by 8000 V for 2hrs (40,000 volt-hours) in linear voltage slope, and was ramped to 1000 V (30,000 volt-hours) for rapid slope (at stages of maximum 50 µA to a minimum of 2µA). IPG strips were frozen in -80°**C** until 2-DE performed. Prior to 2-DE, IPG strips were thawed in room temperature for 10 min and were soaked for 20 min in IPG equilibration buffer I (6 M urea, 2%SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 2% DTT) followed by 20 min in IPG equilibration buffer II (pH 8.8) (20% glycerol, and 2% DTT and 2.5% iodoacetamide).

Second Dimension Electrophoresis and Gel Staining

Equilibrated IPG strips were laid on top of the gels [Criterion Precast Gels (Tris-HCl, 8-16% resolving, 4% stacking) (Biorad, Hercules, CA, USA)], 8µl precision standard marker was loaded in the well to co-electrophores with the samples, and sealed in place using 0.5% low melting point agarose in 25 mM Tris, 192 mM glycine, 0.1% SDS, and a trace of BPB. Second dimension gels were electrophores at 175 Volts for 90 min at 4^0C. After electrophoresis, the gels were washed in three 10 min changes of 200 ml distilled water, and placed in a fixing solution (40% methanol, 10% acetic acid, and 50% H_20) for 1 hr. The gels were subject for two, 5 min rinsed with distilled water. The gels were washed again in a solution containing 50% ethanol, 50% H_2O overnight at 4°**C**, followed by rinsing with H_2O for here times. Gels were placed in 200 ml Colloidal Coomassie Blue G250 (Bio-Rad's Bio-Safe) (Biorad, Hercules, CA, USA) staining solution until the protein spots were adequately stained. The stained gels were rinsed three times, and washed overnight with H_2O. During all the steps for gel staining, the gels were subject to medium shake in an orbital shaker. All the gels were stored in 2% acetic acid solution in water and wrapped with aluminum foil until they were analyzed.

Image Analysis

The 2-D gels of cyanide induced and uninduced fractions were analyzed with PDQuest 2-D analysis software (7.4.0) package and images were filtered to minimize background, and protein spots were identified using automated spot detection wizard, combined with manual editing to remove artifacts. Interested protein spot was confirmed using 3D analysis and the individual spots were compared between gels from uninduced and induced fraction. Molecular masses of the proteins were determined by comparing with the co-electrophoresed Precision Plus Protein Kaleidoscope standard marker (Biorad, Hercules, CA, USA) ranging from 10 kDa to 250 kDa. Iso-electric points were determined based on the linearity of the IPG strip. The interested protein spots were excised (1.0 mm^3 cubes) manually or using EXQuest Spot Cutter form Bio-Rad, and, kept at -20°C, in silianized eppendorf tubes, until further analysis is performed.

In-gel Trypsin Digestion and Peptide Extraction

Protein spots were digested and extracted from gels using a modified method described Vergote *et. al.,* 2006. The excised protein was agitated and washed for 2, 30 min interval in ddH_2O. Samples were destained three times, by agitating in a 200 µl of destaining solution (25 mM ammonium bicarbonate in 50% acetonitrile) for 10 min at 37°C. The supernatant was removed using gel-loading pipette tips and treated followed by two, 200 µl of 90% acetonitrile for 10 min each, where by the gel pieces are shrunk and turned opaque/white, in 100 µl deactivated glass inserts from Agilent Technologies. These opaque white gel pieces were dried in a Speedvac for 30 min. The dried gel pieces were then treated with 25 µl of 25mM dithiothreitol (DTT) in 25 mM NH_4HCO_3, and incubated at 60°C for 1h. The supernatant was removed and the gel pieces were subjected to the addition of 25 µl of 55 mM iodoacetamide and the mixture was incubated at room temperature for 45 min in the dark. The supernatant was discarded, and the gel

pieces were dried for 30 min in Speedvac. The dried gel pieces were treated with 10 µl of 12.5 ng/ µl trypsin (Promega, Madison, WI, USA) in 10 µl of 50 mM ABC and incubated in ice until the gel pieces were fully swelled (30–40 min). The fully swollen gel pieces were further mixed with 5-15 µl each of trypsin (12.5 ng/ µl) and 50 mM NH_4HCO_3 so that all the gel pieces were covered completely, and incubated overnight (16-18 hrs) at 37^0C. The supernatants from the trypsin-digested mixtures were collected in separate 100 µl deactivated glass inserts from Agilent Technologies. The gel pieces were subjected to agitation for 10 min, sonicated for 3 min and centrifuged at top speed for 3 min in 25 µl of 25 mM ABC, twice in 25 µl of 50% ACN/5% formic acid in deionized H_2O, 25 µl of 50% ACN/Isopropanol and 2% formic acid, and finally in 15 µl of 95% ACN/5% formic acid. The protein extract was frozen in liquid N_2 and lyophilized. The lyophilized protein flakes were resuspended in 8 µl of 50% ACN, 45% water, and 5% formic acid and briefly sonicated. Alternatively, protein extract was dried to the volume of around 10 ul using speed vacuum machine (Savant, Minnesota, USA). The protein solution was kept in -20°C until mass spectrometric analysis.

Electro Spray Tandem Mass Spectrometry (ESI-MS/MS)

Eight micro liters (8 µl) of the resuspended samples was automatically injected in capillary LC-MS (ESI-MS/MS). For the liquid chromatography, an Agilent 1100 binary pump (Agilent technologies, Palo Alto, CA) was used, together with a reversed-phase capillary column, 0.3mm x 150 mm, packed with 3.5 µm C_{18}. The LC method consists of, Solvent A [H_2O/0.1% formic acid (FA)] and Solvent B [(ACN/0.1% FA) in a 0-100% gradient (10 min 10%ACN, 10 min 30%, 20 min 70%, 20 min 100%) with a flow rate of 4ul/min for 60 minutes. The peptides eluted were then analyzed by ESI-MS/MS. The spectrometer was operated in data-dependent mode, automatically switching to MS/MS mode. For each scan, the most intense ions

were sequentially fragmented in the linear trap by collision-induced dissociation using collision gas, and results in MS/MS peaks. The most intense MS/MS peaks, in positive ion mode, were automatically selected after defining an intensity threshold. Monoisotopic masses from the tryptic digests were identified using available protein databases which are described in the later part of Materials and Methods.

Isolation of DNA

Genomic DNA from Pf11764 was prepared using a Wizard® Genomic DNA Purification Kit (Promega, Wisconsin, USA). Plasmid DNA from *E. coli* strains was prepared using a Wizard® Plus Miniprep DNA Purification System (Promega, Wisconsin, USA).

PCR Methodology

The general strategy for PCR amplification was carried out according to the manufacturer specifications included with a DNA Walking ACP (Annealing Control Primer)-PCR™ kit from Seegene® (Rockville, MD, USA). Two sets of three PCR reactions were carried out using target specific oliginucleotide primers (TSP1, TSP2, ……..TSP6) (Table 4) designed from known Pf11764 DNA sequence and random primers (DW-ACP) provided with Seegene® kit for amplification each of the upstream *hyp*1 region and downstream region of *nit* gene. In the first PCR reaction one of the four DNA Walking-Annealing Control Primers (DW-ACP) from Seegene® (Rockville, MD, USA) with the first target-specific primers was used to amplify the target region from the template. The second PCR (the first nested PCR) then used the DW-ACPN primer and the 2nd target specific primers to amplify the target from the first PCR reaction. A third PCR (the second nested PCR) was repeated using Universal Primer (Seegene®) and the 3rd target specific primers and the second PCR product as the template.

The first PCR reaction was performed independently in four individual tubes using primer pairs that were a combination of the 10 μM TSP 1/TSP 4 (Table 4) primer with 2.5 μM one of the DW-ACP 1 (5′-ACP-AGGTC-3′), DW-ACP 2 (5′-ACP-TGGTC-3′), DW-ACP 3 (5′-ACP-GGGTC-3′) or DW-ACP 4 (5′-ACP-CGGTC-3′) primers. The following reagents were added to PCR tubes on ice: 50 ng whole genomic DNA, 4 μl DW-ACP (2.5 μM) (one of DW-ACP 1, 2, 3, and 4), 1 μl TSP 1/ TSP 4 (10 μM), 19 μl distilled water, 25 μl Seegenes's 2X SeeAmp™ ACP™ Master Mix II giving total reaction volume of 50 μl. Tubes were placed in a preheated (94^0C) thermal cycler (BioRad®, CA, USA) and following program was used for the PCR reaction. Segment 1: denaturation at 94^0C for 5 min, Segment 2: 42^0C for 1 min, Segment 3: 72^0C for 2 min, Segment 4: 25 cycles of 94^0C for 40 sec, 55^0C for 40 sec, and 72^0C for 1 min 20 sec, Segment 5: 72^0C for 7 min. PCR products were purified using a PCR purification Kit (QIAGEN, CA, USA) to remove the DW-ACP and TSP 1/TSP 4 primers used in the first PCR reaction.

In second PCR reaction, the following reagents were added to PCR tubes on ice: 2 μl Purified first PCR products, 1 μl DW-ACPN (10 μM) (5′-ACPN-GGTC-3′), 1 μl TSP 2/ TSP 5 (10 μM) (Table 4), 6 μl distilled water, 10 μl Seegenes's 2X SeeAmp™ ACP™ Master Mix II giving total reaction volume of 20 μl. Tubes were placed in a preheated (94^0C) thermal cycler (BioRad®, CA, USA) and immediately following program was run for the PCR reaction. Segment 1: denaturation at 94^0C for 3 min, Segment 2: 35 cycles of 94^0C for 40 sec, 60^0C for 40 sec, and 72^0C for 1 min 20 sec, Segment 3: 72^0C for 7 min.

Before commencing 3rd PCR reaction, the second PCR products were diluted 10-fold by adding distilled water to avoid smearing problem in 3rd PCR. In third PCR reaction the following reagents were added to PCR tubes on ice: 1 μl of 10-fold diluted second PCR products, 1 μl

Universal primer (10 µM) (5′-TCACAGAAGTATGCCAAGCGA-3′), 1 µl TSP 3/ TSP 6 (10 µM) (Table 4), 7 µl distilled water, 10 µl Seegenes's 2X SeeAmp™ ACP™ Master Mix II giving total reaction volume of 20 µl. Tubes were placed in a preheated (94°C) thermal cycler (BioRad®, CA, USA) and following program was run. Segment 1: denaturation at 94^0C for 3 min, Segment 2: 35 cycles of 94°C for 40 sec, 65°C for 40 sec, and 72°C for 1 min 20 sec, Segment 3: 72°C for 7 min. After 3rd PCR reaction 5-10 µl of the PCR product was run on 0.8%-1% agarose gel using TAE buffer (0.04 M Tris-acetate; 0.001 M EDTA Na_2-salt, pH 8.0) at 60V until the bromophenol blue loading dye was ¾ down the gel. 1 kb DNA ladder (New England BioLabs, Inc., MA, USA) was used as standard. The gel was stained with ethidium bromide (EtBr) (0.2 µg/ml) and photographed using the DigiDoc-It® Imaging System (UVP Inc., CA, USA).

DNA Sequencing

The 3rd PCR product from gene walking experiment was cloned in *E. coli* DH5α-$T1^R$ (Table-1) using pCR4®-TOPO TA cloning vector (Table-1). Plasmid DNA was extracted from the positive clones (Cells were grown overnight in Luria-Bertini broth/ $Kan^{100µg/ml}$) and suspended in sterile water. DNA was digested using restriction enzyme EcoRI and separated by electrophoresis on a 0.8%-1% agarose gel. The insert was sequenced from MWG Biotech (MWG Biotech, Inc., NC, USA) using the dideoxy chain termination method (Sanger et al., 1977). Both M13 Reverse (-29) and M13 Forward (UNI) primers were used for sequencing on both DNA strands.

Cloning and Expression of *hyp1*

The candidate *hyp1* gene was amplified from *P. fluorescens* genomic DNA using forward primer (*hyp1_f*) and reverse primer (*hyp1_r*) (Table 5). To amplify *hyp1* PCR product the

following PCR conditions were used: Segment 1: denaturation at 94^0C for 3 min, Segment 2: 35 cycles of 94^0C for 40 sec, 65^0C for 40 sec, and 72^0C for 1 min 20 sec, Segment 3: 72^0C for 7 min. After PCR reaction 5-10 µl of the PCR products run on 0.8%-1% agarose gel. 1 kb DNA ladder (New England BioLabs, Inc., MA, USA) was used as a standard. The gel was stained with ethidium bromide (EtBr) (0.2 µg/ml) and photographed using the DigiDoc-It® Imaging System (UVP Inc., CA, USA). An ~ 0.5 kb PCR product was cloned into pET101/D-TOPO® cloning vector to produce the pET101/D-TOPO^R :: *hyp1* recombinant (pGK1) (see Fig. 7) containing a C-terminal six-His tag. Ampicillin resistant transformants of *E. coli* TOP10 (Table-1) cells were screened by colony PCR for the evidence of *hyp1* using same methodology said above. To further verify the existence of the *hyp1* insert, pGK1 was digested with *Sac*I and *Xba*I restriction enzyme's and analyzed on agarose gel. This produced pGK1 was sequenced from MWG Biotech (MWG Biotech, Inc., NC, USA) using both T7 and T7Term primers for bidirectional sequencing to confirm its identity.

For expression, cells of *E. coli* BL21 Star™ (DE3) strain transformed with pGK1 (DK1290) and were cultured overnight at 37°C with agitation @ 250 rpm in LB broth containing 100 µg/ml ampicillin. DK1290 cells containing the pGK1 were inoculated (2% v/v inoculum) to a 10 ml of same LB media and grown for ~2 hours. When cultures reached an absorbance of about ~ 0.5 at 600 nm, isopropyl β-D-1-thiogalactopyranoside was added to a concentration of 1 mM to induce expression of the recombinant protein. Culture was grown for an additional 5 hours and at various interval samples from the culture were withdrawn. After that the cells were harvested and washed in 50 mM Na_2HPO_4-KH_2PO_4 (pH 7.0) buffer before being stored at -80°C until use. Cellular extracts of *E. coli* BL21 Star™ (DE3) cells were prepared according to the user manual (Invitrogen, CA, USA) using lysis buffer (50 mM potassium phosphate, pH 7.8; 400 mM

NaCl, 100 mM KCl; 10% glycerol, 0.5% Triton X-100, 10 mM imidazole). Pellet and supernatant fractions were stored at –80 °C until further use. The over expressed protein (Hyp1) was analyzed by ESI-MS/MS for its identity followed by trypsin digestion and 16% SDS-PAGE. All the cloning and over expression reaction steps were performed according to the user manual (Invitrogen, CA, USA).

Sequence Analysis

Monoisotopic fragment masses obtained from ESI-MS/MS were identified via the MASCOT search algorithm public database (http://matrixscience.com). Search options allowed were only specific tryptic cleavage and included one missed cleavage site. The sensitivity of these approaches was determined using standard protein (BSA). The corresponding proteins were analyzed in bacteria databases using the Basic Local Alignment Search Tool (BLAST) service of the National Center for the Biotechnology Information/National Institure of Health (NCBI/NIH) server (http://www.ncbi.nlm.nih.gov/) and ExPASY (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://www.expasy.ch/).

NCBI/NIH server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used throughout the cloning and sequencing of the *hyp1* as diagnostic tools for matching the obtained sequencing results with information resources for similarities with other known nucleotide or amino acid sequences (Altschul et al., 1997). ExPASY (http://www.expasy.ch/) was used for analysis of predicted Hyp1 protein sequences and to determine its theoretical isoelectric point and mass value.

CHAPTER III

RESULTS

The 17 kDa Polypeptide Made by Pf11764 in Response to Cyanide is a Protein with No Known Function Found in a Select Number of Bacteria

In an effort to determine whether a similar size protein as that reported to be present in cyanide grown cells of Pf11764 by Harris and Knowles (1983b) could be detected in cells induced for CNO and FDH (Kunz et al., 1994; Fernandez et al., 2004), cell extracts (150,000 x g, cytosolic cell fraction) were analyzed by SDS-PAGE. In Figure 1 it can be seen that indeed a protein of approximate 19 kDa was present in cells that were induced with cyanide but not in cells that were not induced. Despite the slight difference in apparent size between the protein detected in our experiments and that reported by Harris and Knowles (1983b), it was hypothesized that the two were probably one and the same. To identify this protein, the visible band was cut out from SDS gels and protein subjected to trypsin digestion prior to analysis of peptide fragments by tandem ESI-MS/MS. These experiments yielded on average four separate protein hits the identities of which are shown in Table 2. This implied that the single protein band extracted from gels was either representative of more than one polypeptide, or that there existed sufficient similarity in the peptides from all such that it was not possible to identify a single target species. To distinguish between these possibilities cell extracts were subjected to 2-dimensional gel electrophoresis in order to more adequately resolve proteins. It was decided to carry out an initial 2D protein gel separation at an acidic to neutral range pH (4-7) because two of the four proteins detected in the single-band extracted from one dimensional gels had approximately equal molecular weights (17,567 and 17,964 kDa) and eluted at similar pI values

(4.41-5.44). Figure 2 shows the results of this experiment in which a single spot of ~19 kDa mass (pI 5.5) was detected (using the BioRad PDQuest^{TM} 2D gel analysis software) in induced cells. This spot was excised and after trypsin digestion peptides were analyzed by ESI-MS/MS as described for protein bands removed from 1-D gels. In this case a single match for a protein annotated as a hypothetical (unknown function) protein present in the bacterium, *Photorhabdus luminescens* subsp. *laumondii* TTO1 (PluTT01) was returned (Table 3). Because the same protein was also consistently detected on 1-D gels (Table 2), it was concluded that this polypeptide made by Pf11764 in response to cyanide was an ortholog of the Hyp1 protein found in PluTT01. This protein represented the target protein of interest and therefore, further experiments were performed to determine its possible biological function.

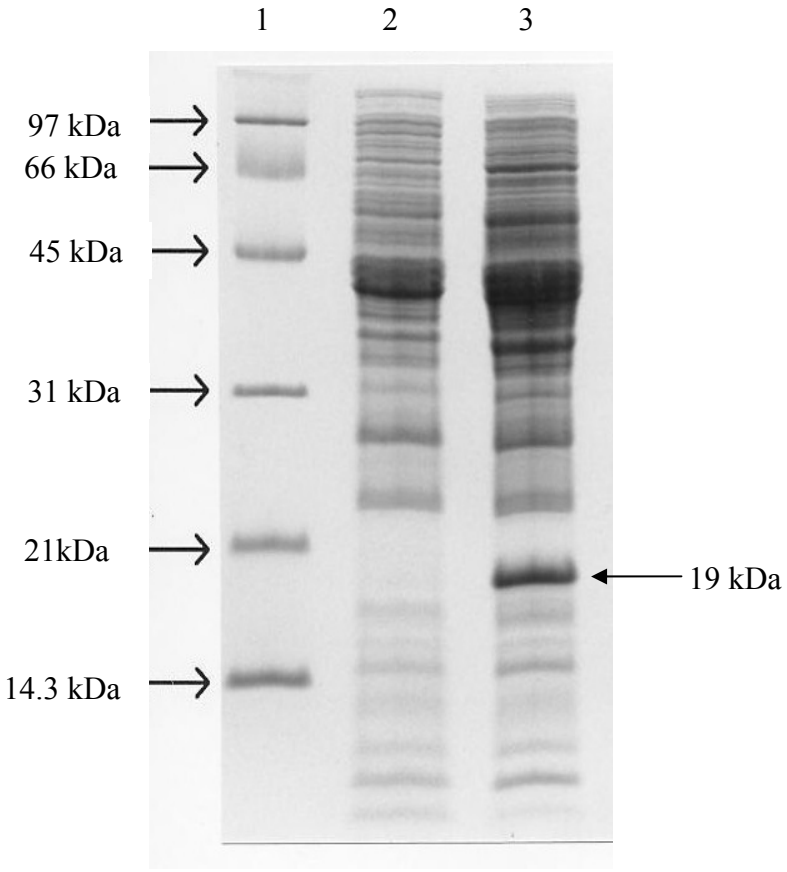

Figure 1: SDS-PAGE (16%) of Pf11764 cell extracts. Lane 1, protein molecular weight standards (5 µg); lane 2, cell extract from uninduced cells (20 µg); lane 3, cell extracts from induced cells (20 µg).

Table 2: ESI-MS/MS protein identity matches with a cyanide up regulated ~19 kDa polypeptide in *P. fluorescens* 11764.

Gene Bank No	***Protein Match in MASCOT MS/MS ion search***	***MASCOT Ion Score***	***No. of peptide matches***	***% coverage***	***Nominal mass (Mr), Da***	***Calculated pI value***
gi|77457799	*CheW protein [Pseudomonas fluorescens PfO-1]*	*178*	*4*	*31%*	*17964*	*4.41*
gi|26987191	*30S ribosomal protein S7 [Pseudomonas putida KT2440]*	*163*	*5*	*37%*	*17567*	*10.15*
gi|91981672	*ribosomal protein [Pseudomonas sp. F96.27]*	*163*	*5*	*42%*	*15177*	*10.13*
gi|37525199	*hypothetical protein plu1232 [Photorhabdus luminescens subsp. laumondii TTO1]*	*95*	*7*	*25%*	*17804*	*5.44*

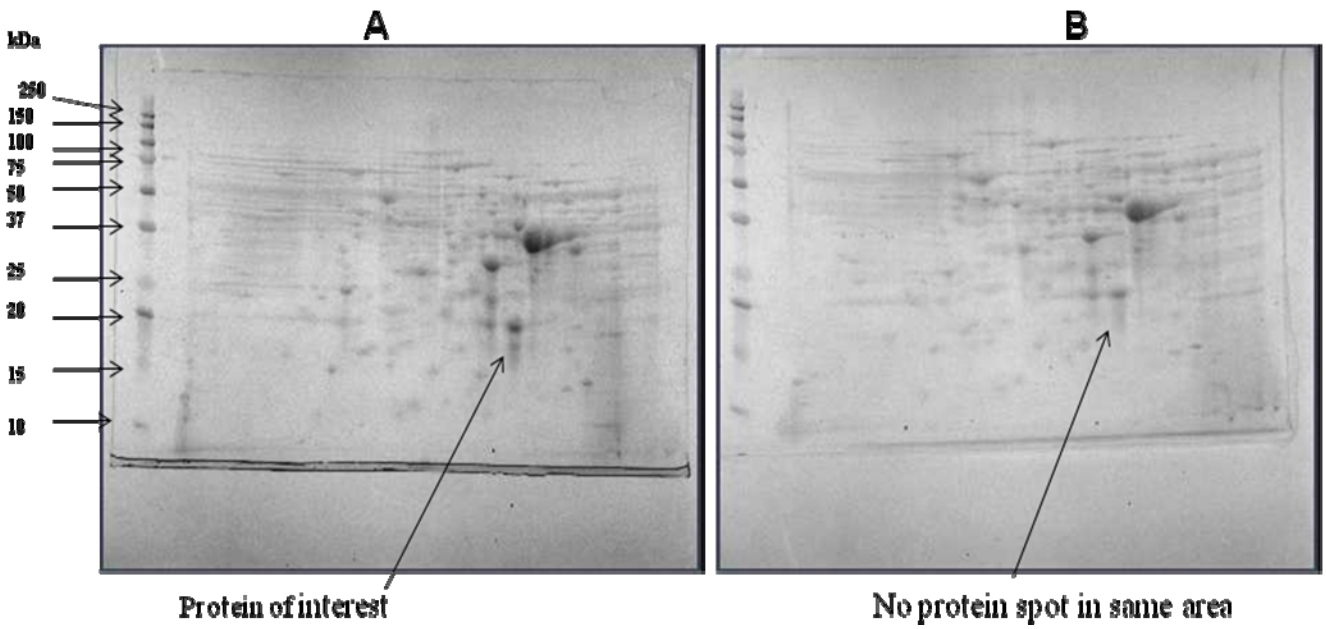

Figure 2: Two-dimensional gel electrophoresis of cell extract protein (250 µg) from cyanide-induced (Panel A) and uninduced cells (Panel B) of *P. fluorescens* 11764.

Table 3: Single identity match for the induced 19 kDa polypeptide made by *P. fluorescens* 11764 following resolution by 2D gel electrophoresis & peptide analysis by mass spectrometry.

Gene Bank No	***Protein Match in MASCOT MS/MS ion search***	***MASCOT Ion Score***	***No. of peptide matches***	***% coverage***	***Nominal mass (Mr), Da***	***Calculated pI value***
gi|37525199	*hypothetical protein plu1232 [Photorhabdus luminescens subsp. laumondii TTO1]*	*95*	*7*	*25%*	*17804*	*5.44*

The Pf11764 Hyp1 Protein is Part of a Unique Gene Cluster That Includes The Putative Pf11764 CynD Nitrilase

As described in the Introduction, the CynD protein is one of four enzymes thought to be responsible for cyanide degradation by Pf11764. As part of an effort to try and determine the relatedness of this enzyme to other CynD enzymes described in bacteria, a collaboration between our laboratory and that of Dr. Michael Benedik at Texas A&M University was undertaken to identify the gene coding this enzyme in Pf11764. Dr. Benedik has published extensively on the properties of bacterial CynD enzymes being the first to describe the cloning of such an enzyme from a bacterium (*Bacillus pumilus*) (Jandhyala et al., 2003). Because preliminary studies by Dr. Ruby Fernandez had demonstrated that the Pf11764 CynD enzyme shared catalytic and molecular properties (Fernandez and Kunz, 2005) with that of the enzyme from *Bacillus pumilus* ($CynD_{Bp}$), it was hypothesized that the Pf11764 CynD enzyme ($CynD_{Pf11764}$) was a likely ortholog of $CynD_{Bp}$ (and a homologue also from *P. stutzeri*, Watanabe et al., 1998) enzyme. Therefore, initial efforts to identify the gene from Pf11764 by Dr. A. Srivastava in our laboratory and by Dr. Benedik at Texas A&M relied on the use of $CynD_{Bp}$ oligonucleotides to PCR amplify the putative Pf11764 homologue. These efforts on the part of both investigators were unsuccessful suggesting that the enzyme from Pf11764 was not a direct homologue of the *Bacillus pumilus* enzyme. Dr. Benedik subsequently constructed new oligonucleotides and managed ultimately, to obtain a product whose sequence (441 bp) indeed returned a hit for a nitrilase enzyme. Because nitrilase genes (*nit*) are common and the sequence for the Pf11764 *nit* DNA shared only partial homology with $CynD_{Bp}$, relatively little attention was paid to these findings until it was discovered that the top hit returned from a $BLAST_x$ search of the $nit_{Pf11764}$ sequence was that also of a Nit enzyme from PluTT01 as shown for Hyp1 as described in the

first part of the results. Further bioinformatics analysis revealed that the corresponding *hyp1* and *nit* genes in PluTT01 reside next to each other and are each members of a cluster of genes referred to as Nit1C whose function is unknown (Podar et al., 2005). Figure 3 shows the arrangement of this gene cluster in PluTT01 (*hyp1* and *nit* reside at loci Plu_1232 and Plu_1231, respectively). Having available 441 bp of Pf11764 *nit* sequence (Nit_{11764}) it was now possible to conduct experiments to determine whether *hyp1* and *nit* genes in Pf11764 also resided next to each other as is the case for the gene in PluTT01 (and also for Nit1C found in other bacteria). To accomplish this three target specific oligonucleotide primers (TSP1, TSP2, TSP3) (Table 4) based on the known $nit_{Pf11764}$ DNA sequence were constructed and used to PCR amplify the upstream *hyp*1 region (Figure 4). This was accomplished using a gene walking strategy as shown in Figure 4 (see details in Materials and Methods) in collaboration with Dr. J-H Lee, a visiting scientist in our laboratory. Several PCR products were obtained the largest of which (~0.7 kb) was cloned into plasmid vector pCR4®-TOPO and the sequence confirmed as that of *hyp*1. Similarly, Dr. Lee constructed another three target specific oligonucleotide primers (TSP4, TSP5, TSP6) (Table 4) which were designed to PCR amplify the downstream *nit* region (Fig 4). In this case a ~1.1kb PCR product was obtained and the sequence, which contained the entire downstream region of *nit* was also confirmed. Compiling the total sequence data obtained allowed for the complete physical structure of this region to be formulated, and this is illustrated in Figure 5. That the deduced amino acid sequence of $Hyp1_{Pf11764}$ was correct was further confirmed by an independent experiment which showed that the first nine amino acids (MPNVDIAHY) determined by Edman degradation of the polypeptide purified from SDS gels were the same as that determined from the nucleotide sequence.

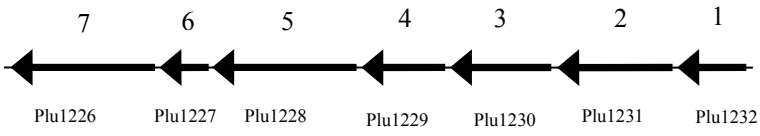

Figure 3: Organization of Nit1C cluster in *Photorhabdus luminescence subsp. laumondii TT01* (adapted from Podar et al., 2005). (1) Hyp1, hypothetical protein (2) Nit, Nitrilase (3) SAM, radical S-adenosyl methionine superfamily member, (4) GNAT, acetyltransferase, (5) AIR synthase, member of the large 5'-phosphoribosyl-5-aminoimidazole synthase-related proteins, (6)Hyp2, hypothetical protein, and (7) FP, possible flavo protein monooxygenase.

Table 4: Oligonucleotide primers used to PCR amplify* *hyp*1 and *nit* DNA from Pf11764.

Primer name	Target-specific primer sequence (5'-3')	Purpose
TSP1	5' CTGCACGAACGAGAAGTAGGG 3'	For amplification of *hyp*1 and its upstream flanking region
TSP2	5' CTAACTGCAAGCGTTGTCGGC 3'	
TSP3	5' CGAGTAAAGCACGGGACTGCACTG 3'	
TSP4	5' GATGGCGGCACAATCTACAAC 3'	For amplification of *nit* and its downstream flanking region
TSP5	5' CCAGCACCGACGCAAGATAACC 3'	
TSP6	5' GTCTGGGGCCGAAGGCGAC 3'	

***** For reverse oligonucleotide primers, Seegenes's DNA walking primers (Rockville, MD, USA) were used.

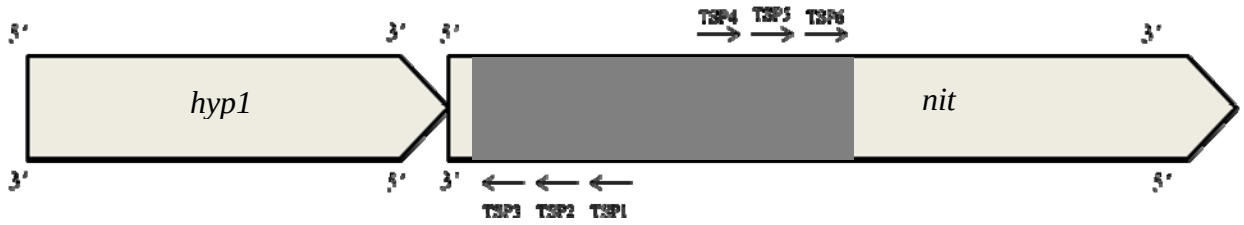

Figure 4: Physical arrangement of *hyp1 & nit* Pf11764 open reading frames and region specific primer construction for gene walking sequence analysis. The 441 bp *nit* DNA sequence from Dr. Benedik is shown as a deep shaded region.

```
                                                                  -10
-59 TGTAACCGATCGGCCGATCCCGGCCATCGATAACAGCAAGTACTACTGGAGACGACGACGAC

  1 ATG CCC AAT GTG GAC ATT GCC CAC TAT CAA GAC GGC GAC TTT TTG
    M   P   N   V   D   I   A   H   Y   Q   D   G   D   F   L

 46 GTC AAC TAC GAA GAA AAG GTC TTC GAG GAC GTC AAG GCC GCA CCC
    V   N   Y   E   E   K   V   F   E   D   V   K   A   A   P

 91 GGT GAC AAG GCC CTG TTG ACC TTC CAC ACG ATC GCC TTC GAA GGC
    G   D   K   A   L   L   T   F   H   T   I   A   F   E   G

136 TCC ATT GGC CTG GTC AAC ATG CTG CAA GCC AAG CGC CTG CTG CGC
    S   I   G   L   V   N   M   L   Q   A   K   R   L   L   R

181 AAA GGT TTT GAA ACC AAG ATC CTG CTC TAC GGG CCA GGC GTG CAA
    K   G   F   E   T   K   I   L   L   Y   G   P   G   V   Q

226 CTT GGC GTG CAA CGC GGC TTC CCC ACC TTG GGC GCC GAG GCC TTC
    L   G   V   Q   R   G   F   P   T   L   G   A   E   A   F

271 CCT GGC CAC TTG GCC GTC AAT AAC CAG ATC AAG GCA TTC ATG GCT
    P   G   H   L   A   V   N   N   Q   I   K   A   F   M   A

316 GAA GGC GGT GAA GTC TAT GCC TGC CGC TTT GCC TTG CAG GCG CTG
    E   G   G   E   V   Y   A   C   R   F   A   L   Q   A   L

361 TAT GGG CAA ACC GAA AAA GCC CTG ATC GAA GGC ATT CGC CCA ATC
    Y   G   Q   T   E   K   A   L   I   E   G   I   R   P   I

406 AAC CCT CTG GAT GTC ATG GAC CTG CGT CTG CTG ATG CGT CGT GAG
    N   P   L   D   V   M   D   L   R   L   L   M   R   R   E
                                                  483
451 GGC GCG ATG ATC ATC GAT ACC TGG ACT GCC TGA GTCGCGGCCCACCCG
    G   A   M   I   I   D   T   W   T   A stop
                                                  -10
CTCCGCGCAGGAAGCGGGCGGGCCTGCAAAGACCTTCAGCCTCCCTCCGCCGCCCCAAGGAATTACCCC

  1 ATG CCC GTT ATT CGC GCC GCC GCC GTG CAG TGC AGT CCC GTG CTT
    M   P   V   I   R   A   A   A   V   Q   C   S   P   V   L

 46 TAC TCG CGC CAG GCA ACC GTC GAC AAA CTC TGC CGC CAG CTG CTG
    Y   S   R   Q   A   T   V   D   K   L   C   R   Q   L   L

 91 GAA CTG GGC CGA CAA CGC TTG CAG TTA GCT GTC TTT CCG GAA ACC
    E   L   G   R   Q   R   L   Q   L   A   V   F   P   E   T
```

```
136 GTG GTG CCG CAC TAC CCC TAC TTC TCG TTC GTG CAG CCG CCC TGT
     V   V   P   H   Y   P   Y   F   S   F   V   Q   P   P   C

181 GCC ATG GCC GGG CAA CAC CTC ATG CTG CTC GAA TAC TCC GTC ACC
     A   M   A   G   Q   H   L   M   L   L   E   Y   S   V   T

226 GTG CCC TCG GCC GTC ACC CGG CAG ATC GCC GAA CCC TGC CGC GAA
     V   P   S   A   V   T   R   Q   I   A   E   P   C   R   E

271 GCC AAC CTC GTC ACC TGC ATC GGT GTC AAC GAA CGC GAT GGC GGC
     A   N   L   V   T   C   I   G   V   N   E   R   D   G   G

316 ACA ATC TAC AAC GCT CAA CTG CTG TTC GAC GCC GAC GGC ACG CTT
     T   I   Y   N   A   Q   L   L   F   D   A   D   G   T   L

361 ATC CAG CAC CGA CGC AAG ATA ACC CCC ACC TAT CAC GAG CGC ATG
     I   Q   H   R   R   K   I   T   P   T   Y   H   E   R   M

406 GTC TGG GGC CAG GGC GAT GGC TCC GGT CTG CGC GCT ACC GAC AGC
     V   W   G   Q   G   D   G   S   G   L   R   A   T   D   S

451 GCC GTC GGC CGC ATC GGC GCC CTC GCT TGT TGG GAA CAT TAC AAC
     A   V   G   R   I   G   A   L   A   C   W   E   H   Y   N

496 CCG CTG GCG CGC TAC GCG CTG ATG GCC GAT GCA GAA CAG ATT CAC
     P   L   A   R   Y   A   L   M   A   D   A   E   Q   I   H

541 GCA GCG ATG TTC CCC GGT TCC CTG GTC GGT CAG ATC TTC GCT GAT
     A   A   M   F   P   G   S   L   V   G   Q   I   F   A   D

586 CAG ATC GAA GTC ACC ATC CGC CAT CAC GCC CTG GAA AGC GGC TGT
     Q   I   E   V   T   I   R   H   H   A   L   E   S   G   C

631 TTT GTG GTC AAC GCC ACT GCC TGG CTG GAT GCT GGG CAA CAG GCA
     F   V   V   N   A   T   A   W   L   D   A   G   Q   Q   A

676 CAA ATC ATG GCC GAC ACA GGT TGT GCC ATC GAA CCT ATC TCA GGT
     Q   I   M   A   D   T   G   C   A   I   E   P   I   S   G

721 GGT TGC TTC ACG GCC ATC GTT TCG CCG GAA GGC AAG CTG CTC GCG
     G   C   F   T   A   I   V   S   P   E   G   K   L   L   A

766 CAG ATG ACC GAG GGC GAA GGG GTG ATC ATC GCC GAC CTC GAC TTT
     Q   M   T   E   G   E   G   V   I   I   A   D   L   D   F

811 GCC TTG ATC GAC AAG CGT AAA CGC ATG ATG GAT TCA GTC GGC CAT
     A   L   I   D   K   R   K   R   M   M   D   S   V   G   H

856 TAC AGC CGC CCG GAA TTG CTT AGC CTG CAG ATC GAC CGC CGA GTC
     Y   S   R   P   E   L   L   S   L   Q   I   D   R   R   V
```

```
                                                                  945
901 ACG GCA CAT GTG CAT GAG CGT GGC GAG GCT TGC GAA CAT GAA TAG CACT
     T   A   H   V   H   E   R   G   E   A   C   E   H   E stop

   CACTGCCTGGCTGGATGCTGGGCAACAGGCACAAATCATGGCCGACACAGGTTGTGCCATCGA
   ACCTATCTCAGGTGGTT
```

Figure 5: Nucleotide and deduced amino acid sequences of Pf11764 *hyp1* and *nit* DNA. The sequence of the two contiguous genes is shown in the 5′- 3′ direction with their flanking regions. The sequence shown includes the 480-bp open reading frame for *hyp1* and 942-bp open reading frame for *nit*. The putative ribosome binding site at -10 is shown for each in boldface type and underlined.

Cloning of *hyp1* for Expression in *E. coli*

To determine the biochemical function of Hyp1 an attempt was made to clone the gene for over expression and characterization of the protein. To accomplish this, oligonucleotide primers (Table 5) were constructed from *hyp1* DNA and used to amplify the entire *hyp1* ORF. These experiments produced a 483bp PCR product (Figure 6) which was directionally cloned into the expression vector, pET101/D-TOPO® (Invitrogen®), yielding plasmid recombinant pGK1 (Fig. 7). The expression of insert DNA in this vector is under the control of a high-level T7 promoter whose activation is regulated by *lacI*. Thus, over production of protein can be manipulated by providing IPTG (isopropyl thiogalctoside) to the cultivation medium (Studier et al., 1990). The directional cloning site further allows for the tagging of the expressed product with six histidine residues facilitating affinity purification (Hochuli et al., 1988; Lu et al., 1996). In order to determine whether Hyp1 was expressed, *E. coli* BL21 Star™ (DE3) One-shot (Invitrogen^{R}) cells (strain designated DK1290) containing pGK1 were inoculated (2% v/v) from an overnight culture and allowed to grow for 2 hours at 37^{o}C (OD_{600} = 0.5) before adding IPTG (1 mM). At various intervals samples from the culture were withdrawn and both cytosolic and pellet (membrane) fractions were analyzed for evidence of an over-expressed protein of predicted 21 kDa (includes 17.8 kDa plus 6 histidine residues and 24 residues encompassing a V5 epitope for Western blotting if preferred) (see Fig 7). As shown in Figure 8 increasing amounts of a ~21 kDa polypeptide became visible on gels with prolonged incubation the maximum amount being produced at approximately 4 hours. Most of the protein, however, was recovered in the membrane (pellet) fraction. This indicated unfortunately, that Hyp1 was produced as an insoluble protein thus complicating bulk isolation & characterization. The presence of inclusion bodies in cells visible on microscopic examination (data not shown) also

gave further evidence of this. Curiously, an additional polypeptide of ~28 kDa whose appearance paralleled that of the putative Hyp1 protein was also observed for reasons that were not understood. To determine the identity of this protein and further verify that the 21 kDa species was indeed Hyp1, protein bands (see Fig. 8) were once again extracted from gels and analyzed by ESI MS/MS. The results of of a Matrix/Mascot database search for peptides matching those detected in the 21 kDa species revealed the strongest homology with those of the PluTT01 Hyp1 protein (the matching peptides are shown in Figure 9 in boldface). These results therefore provided strong evidence that the Hyp1 protein from Pf11764 was indeed capable of being expressed from the recombinant plasmid pGK1 containing the cloned *hyp1*$_{Pf11764}$ insert.

With regards to the second protein (~28 kDa) expressed in parallel with Hyp1, the evidence from a database search of matching peptides indicated that the protein was most identical to class C β-lactamase (E.C. 3.5.2.6) (Table 6). From amino acid sequence analysis this protein corresponds as far as we can tell, to that of the β-lactamase encoded by *bla* on pGK1 (see Fig. 7). Because there is no reason to think that the pGK1 *bla*-encoded β-lactamase should be expressed at such high levels, we hypothesize that the expression of Hyp1 by the recombinant is somehow influencing *bla* gene expression.

Table 5: Oligonucleotide primers used to clone *hyp*1.

Primer name	Primer sequence (5'-3')
Forward primer (*hyp1_f*)	5' CACCATGCCCAATGTGGACATTGCCCAC 3'
Reverse primer (*hyp1_r*)	5' GGCAGTCCAGGTATCGATGATCAT 3'

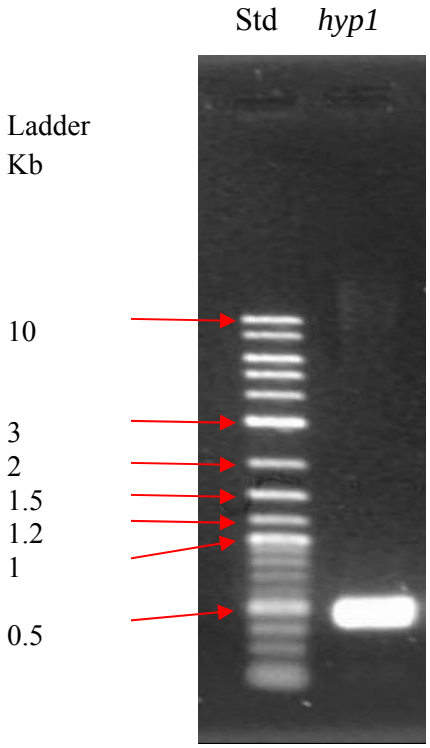

Figure 6: 1% agarose gel of PCR amplified *P. fluorescens*11764 *hyp1*.

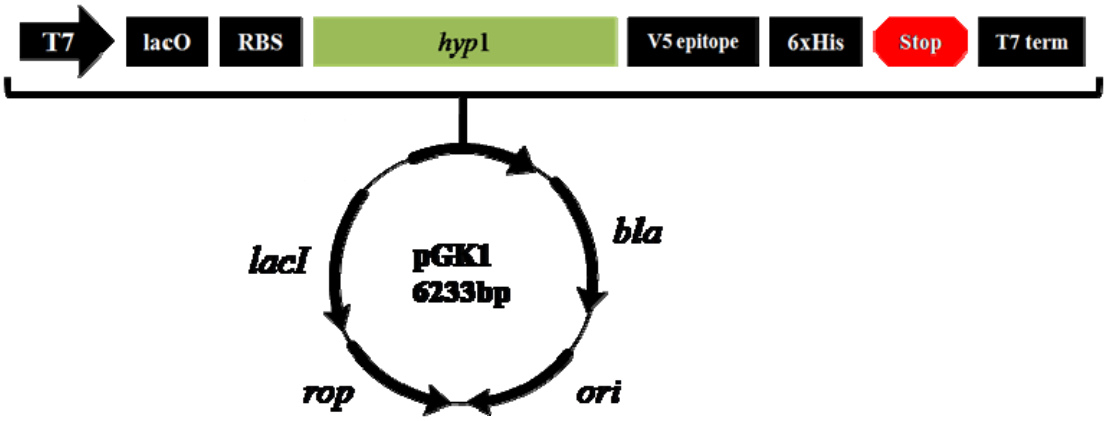

Figure 7: Schematic representation of the pET101D::*hyp1*$_{Pf11764}$ recombinant designated pGK1.

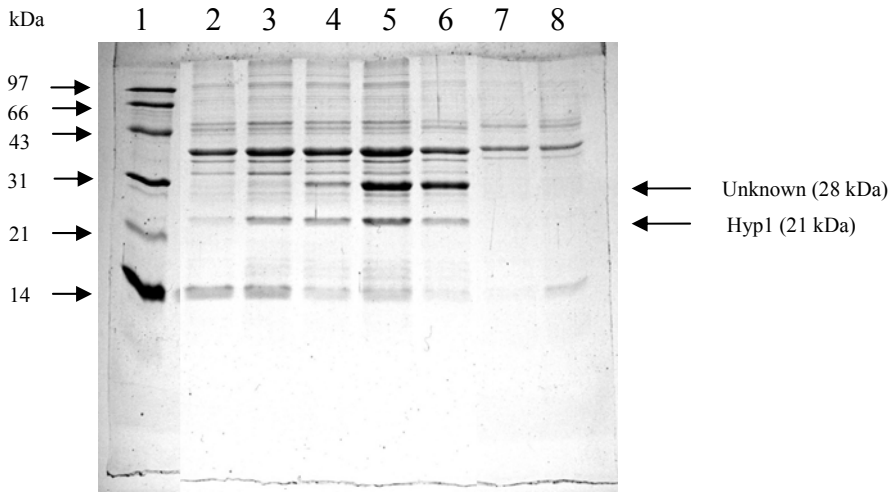

Figure 8: SDS-PAGE (16%) analysis of cell lysates (pellet) fractions from cells of DK1290 following 1, 2, 3, 4 and 5 h induction with IPTG. Cells were cultivated for 2 h at which time IPTG was added and samples removed for SDS-PAGE. Lanes 7 and 8 are pellet fractions from DK1200 cells that were not induced with IPTG sampled after 3 and 4 h cultivation, respectively. Lane 1 contains protein molecular weight standards.

1 MPKIDIEQYK DGDFLVDYEE KVFEDVKALP GEKALVTFHT VAFEGSAGLV

51 NVLQAKRLLR KGFETK**ILLY GPGVQLGVQR GFPTLGAEAF PGHLAVNNQL**

101 **K**AFMEEGGEV YACRFALQAF YGQTEKALIP GIRPINPLDV MDLRLLMRRD

151 NALVIDTWTA

Figure 9: Peptide matches (bold face) between recombinant $Hyp1_{Pfl1764}$ protein expressed from plasmid pGK1 and Hyp1 from PluTT01 identified by MASCOT algorithm public database search (http://matrixscience.com).

Table 6: ESI-MS/MS identity matches with a 28 kDa polypeptide expressed in parallel with Hyp1 by recombinant pGK1.

Gene Bank No	***Protein Match in MASCOT MS/MS ion search***	***MASCOT Ion Score***	***No. of peptide matches***	***% coverage***	***Nominal mass (Mr), Da***
gi|2467190	extended-spectrum TEM-type beta-lactamase variant, TEM-52 [*Klebsiella pneumoniae*]	*565*	*16*	*49%*	*31494*
gi|61967136	beta-lactamase TEM-141 [*Enterobacter cloacae*]	*523*	*14*	*43%*	*31496*
gi|43798	extended-spectrum beta-lactamase TEM-3 [*Klebsiella pneumoniae*]	*515*	*15*	*48%*	*31524*

CHAPTER IV

DISCUSSION

The ability of bacteria to use cyanide as a sole nitrogen source for growth has been recognized for some time but the genetic basis of this ability has not been determined. In this study a preliminary observation published by Harris and Knowles (1983a) of an unknown polypeptide produced by cells of Pf11764 grown on cyanide was confirmed and the identity of the protein determined. Based on mass spectrometry analysis of peptides, the unknown polypeptide was identified as a homologue of so-called Hyp1 from the bacterium *Photorhabdus luminscens* subsp *laumondii* (PluTT01). This protein encoded by the *hyp1* gene is a member of gene cluster found in this and a few other bacteria known as Nit1C (Podar et al., 2005). This cluster includes also *nit* which encodes a nitrilase (Nit) enzyme thought to be involved in enzymatic cyanide degradation by Pf11764. Further studies revealed also that the Pf11764 Nit enzyme is a close homologue of a PluTT01 Nit enzyme and established the adjacent location of the two corresponding genes in a possible operon. Other studies by Dr. Jung-Hyan Lee in our laboratory have shown that additional genes (SAM and GNAT) associated with Nit1C also exist in Pf11764. As the Nit1C cluster is unknown to exist in the taxon *Pseudomonas*, this is the first example of its presence in this genus. Besides PluTT01, bioinformatic analysis indicates its presence in various members of the Domain Bacteria (e.g., *Burkholderia, Klebsiella*, *Ralstonia, Gluconacetobacter, Rhizobium*). However, it is not found in the Archae or Eukarya. The physiological function of Nit1C genes is currently unknown.

Because the expression of Hyp1 was elevated in response to cyanide it was first proposed by Harris and Knowles (1983b) that it could be part of the enzymatic machinery required for

cyanide degradation. While studies showed that the appearance of this protein does coincide with the induction of CNO and FDH enzymatic activities, no evidence that Hyp1 is a component of either of these enzymatic systems exists. With regard to $Nit_{Pfl1764}$ enzyme, presumably also upregulated in response to cyanide (given that the two genes are likely to exist in an operon), additional studies are needed to established that the properties of this enzyme conform to those previously described for CynD, found as a component of the CNO complex (Fernandez and Kunz, 2005). Despite this, there is reason to think that both the Pf11764 *hyp1* and *nit* genes (and others of the putative Nit1C cluster) are necessary for cyanide utilization. This hypothesis is based on several lines of reasoning. First, the discovery of *nit* on the Pf11764 chromosome and that of cyanide-degrading Nit-type (CynD) enzyme required for cyanide degradation infers a potential physiological linkage between the two. Second, the terminal gene found in Nit1C clusters is annotated as a flavoprotein oxidoreductase; most closely related to other flavin-dependent monooxygenases. Thus, because CNO is known to function as a monooxygenase (Wang et al., 1994) there is reason to think that the flavoprotein monooxygenase encoded by Nit1C may be the long sought after enzyme responsible for cyanide monooxygenation. Third, Dr. Kunz has shown that at least one other bacterium (*Burkholderia xenovorans* LB400) known to harbor Nit1C on the chromosome can also grow on cyanide. Although additional Nit1C-containing bacteria need to be screened, this suggests a linkage between bacterial growth on cyanide and Nit1C.

With regards to the function of Hyp1, unfortunately its physiological role remains uncertain. However, there is strong reason to think this protein may function as a transcriptional regulator. This hypothesis is based on a number of factors. First, homology searches of amino acid sequence from fragments are most similar to those with motifs represented by nucleotide

transferases, phosphodiesterases, and cyclic GMP (or adenyl) cyclases (see Table 7), all of which are associated frequently with response regulators of two-component signal transduction systems (Stock et al., 1989; Gao et al., 2007). Second, it may be noted that such response regulators (LysR, CheY, GAF) (Ho et al., 2000; Gao et al., 2007) have been reported in several instances to be associated with the upregulation of beta-lactamases in response to certain stress stimuli (Kallipolitis et al., 2003; Hirakawa et al., 2003). This may help to explain why the pGK1 *bla* encoded β-lactamase was consistently over expressed parallel with Hyp1, particularly in response to IPTG addition to the medium. Thus, we hypothesize that $Hyp1_{Pfl1764}$ can somehow influences the expression of vector encoded β-lactamase. While this idea needs to be further tested it provides at least a partial explanation for why the two proteins were consistently observed in recombinant *E. coli* strains under conditions (IPTG – induction) where Hyp1 expression was optimized.

In summary, studies in this thesis have led to the discovery for the first time of a unique gene cluster (NitC) in *Pseudomonas* bacteria. As the physiological role of this gene cluster is unknown, the finding that at least one of the genes (*hyp1*) responds to cyanide as an environmental signal provides the first evidence of its kind to show that these genes are linked to cyanide metabolism. Whether this linkage is directly associated with cyanide degradation (and thus its nutritional utilization as a nitrogen source) or represents a molecular response to the stress imposed by the poisoning effects of cyanide remains to be further established. Further studies in our laboratory aimed at evaluating the effects of *nit* gene deletions on cyanide growth are expected to provide further information on whether this and/or or other Nit1C genes are directly associated with the unique ability of bacteria to grow on cyanide.

Table 7: Peptide fragments of $Hyp1_{Pfl1764}$ showing high homology to bacterial response regulator motifs.

Arbitrary peptides sequence[a]	Annotation	% amino acid identity	Organism
Complete	GTP cyclohydrolase	72%	*Burkholderia vietnamiensis G4*
MPNVDIAH	transcription regulator	87%	Rhodopirellula baltica SH 1
GDFLVNYEEKVF	PAS/PAC domain protein	75%	*Algoriphagus* sp. PR1
GFPTLGAEAFPGHLA	*hrp* regulatory protein HrpS	70%	Pseudomonas syringae pv. actinidiae
LRLLMRREG	two-component system copper resistance phosphate regulon response regulator	88%	*Burkholderia multivorans* ATCC 17616

[a]. Peptides sequenced in $Hyp1_{Pfl1764}$ and highlighted in the following sequence

Hyp1

MPNVDIAHYQDGDFLVNYEEKVFEDVKAAPGDKALLTFHTIAFEGSIGLVNMLQAKRLL
RKGFETKILLYGPGVQLGVQRGFPTLGAEAFPGHLAVNNQIKAFMAEGGEVYACRFALQ
ALYGQTEKALIEGIRPINPLDVMDLRLLMRREGAMIIDTWTA

REFERENCES

Adje, M. D., and Y. Ohta. 1999. Isolation and characterization of a cyanide-utilizing *Burkholderia cepacia* strain. World J. Microbiol. Biotechnol. **15:**699-704.

Agency For Toxic Substances and Disease Registry. 1993. Cyanide toxicity. Am. Family Phys. **48:** 107-114.

Atschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLASAT and PSI-BLAST: A new generation of protein database search programs. Nucleic Acid Res. **25**: 3389-3402.

Blumer, C., and D. Haas. 2000. Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch. Microbiol. **173**:170-177.

Castric, P. A. 1981. The metabolism of hydrogen cyanide by bacteria. In: Vennesland B et al. (eds) Cyanide in biology. Academic Press. London & New York, p. 233-262.

Conn, E.E. 1980. Cyanogenic compounds.Ann. Rev. of plant Physiol. **31**: 433-451.

Cunningham, L., and H. D. Williams. 1995. Isolation and characterization of mutants defective in the cyanide-insensitive respiratory pathway of *Pseudomonas aeruginosa*. J. Bact. **177:** 432-438.

Dolghih, E. 2004. Bacterial cyanide assimilation: Pterin cofactor and enzymatic requirements for substrate oxidation. M.S. thesis. University of North Texas, Denton, TX.

Fawcett, J. K., and J. E. Scott. 1960. A rapid and precise method for the determination of urea. J. Clin. Pathol. **13:**156-159.

Fernandez, R. 2004. Cyanide assimilation in *Pseudomonas fluorescence* NCIMB 11764: Characterization of cyanide oxygenase as a pterin-dependent multicomponent enzyme complex. Ph.D. dissertation. University of North Texas, Denton, TX.

Fernandez, R.F., E. Dolghih, and D.A. Kunz. 2004. Enzymatic assimilation of cyanide via pterin-dependent oxygenolytic cleavage to ammonia and formate in *Pseudomonas fluorescens* NCIMB 11764. Appl. Environ. Microbiol. **70**:121-128.

Fernandez, R. F., and D. A. Kunz. 2005. Bacterial cyanide oxygenase is a suite of enzymes catalyzing the scavenging and adventitious utilization of cyanide as a nitrogenous growth substrate. J. Bacteriol. **187**: 6396-6402.

Finnegan, I., S. Toerien, L. Abbot, F. Smit, and H. G. Raubenheimer. 1991. Identification and characterization of an *Acinetobacter* sp. capable of assimilation of a range of cyano-metal complexes, free cyanide ions and similar organic nitriles. Appl. Microbiol. Biotechnol. **36:**142-144.

Foor, F., and G.M. Brown. 1975**.** Purification and properties of the Guanosine Triphosphate cyclohydrolase II from *Escherichia coli.* J. Biol. Chem. **250:**3545-3551.

Fry, W.E., and R.L. Millar. 1972. Cyanide degradation by an enzyme from *Stemphylium loti*. Arch. Biochem. Biophys. **151**:468-474.

Fuller, W. H. 1984. Cyanide in the environment with particular attention to the soil. In: van Zyl D. (ed.) Cyanide in the environment, (p.19-44). Geotechnical Engineering Program, Colorado State University, Fort Collins, Co.

Gao, R., T. R. Mack, and A. M. Stock. 2007. Bacterial response regulators: versatile regulatory strategies from common domains. Trends. Biochem. Sci. **32**:225-234.

Gorg, A., C. Obermaier, G. Boguth, A. Harder,B. Scheibe, R. Wildgruber, and W. Weiss. 2000. The current state of two dimensional electrophoresis with immobolised pH gradients. Electrophoresis. **21**:1037-1053.

Harris, R. E., and C. J. Knowles. 1983a. Isolation and growth of a *Pseudomonas* species that utilizes cyanide as a source of nitrogen. J. Gen. Microbiol. **129:**1005-1011.

Harris, R. E., and C. J. Knowles. 1983b. The conversion of cyanide to ammonia by extracts of a strain of *Pseudomonas fluorescens* that utilize cyanide as a source of nitrogen for growth. FEMS Microbiol. Lett. **20**:337-341.

Hirakawa, H., K. Nishino, J. Yamada, T. Hirata, and A. Yamaguchi. 2003. Beta-lactam resistance modulated by the overexpression of response regulators of two-component signal transduction systems in *Escherichia coli*. J. Antimicrob. Chemother. **52**:576-582.

Hochuli, E., W. Bannwarth, H. Dobeli, R. Gentz, and D. Stuber. 1988. Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology*. **6:**1321-1325

Homan E. R. 1988. Reactions, processes and materials with potential for cyanide exposure. In: Ballantyne B. and Marrs T. C. (Eds) Clinical and Experimental Toxicology of Cyanides, (pp 1-21). Wright, IOP Publishing Ltd., Bristol U.K.

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

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

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.

Ho, Y. S. J., L. M. Burden, and J. H. Hurley. 2000. Structure of the GAF domain, a ubiquitous signaling motif and a new class of cyclic GMP receptor. EMBO J. 19:5288-5299.

Kallipolitis, B. H., H. Ingmer, C. G. Gahan, C. Hill, and L. Søgaard-Andersen. 2003. CesRK, a two-component signal transduction system in *Listeria monocytogenes*, responds to the presence of cell wall-acting antibiotics and affects beta-lactam resistance. Antimicrob. Agents. Chemother. **47**:3421-3429.

Knowles C. J., A.W. Bunch. 1986. Microbial cyanide metabolism. Adv. Microb. Physiol **27**:73-111.

Knowles, C. J. (1976). Microorganisms and cyanide. Bacteriol. Rev. **40:** 652-80.

Knowles, C. J. 1988. Cyanide utilization and degradation by microorganisms. In Cyanide Compounds in Biology, CIBA Found. Symp. **140**:3-15.

Kunz, D. A., and O. Nagappan. 1989**.** Cyanase-mediated utilization of cyanate in Pseudomonas fluorescens NCIMB 11764. AppI. Environ. Microbiol. **55**:256-258.

Kunz, D. A., O. Nagappan, J. Silva-Avalos, and G. T. Delong. 1992. Utilization of cyanide as a nitrogenous substrate by *Pseudomonas fluorescens* NCIMB 11764: evidence for multiple pathways of metabolic conversion. Appl. Environ. Microbiol. **58:**2022-2029.

Kunz, D. A., C.-S Wang, and J.-L. Chen. 1994. Alternative routes of enzymic cyanide metabolism in *Pseudomonas fluorescens* NCIMB 11764. Microbiology **140**:1705-1712.

Kunz, D. A., R. Fernandez, and P. Parab. 2001. Evidence that bacterial cyanide oxygenase is a pterin-dependent hydroxylase. Biochem. Biophys. Res. Commun. **287**: 514-518.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-685.

Lambert, J. L., J. Ramasamy, and J. V. Paukstells. 1975. Stable reagents for the colorimetric determination of cyanide by modified König reactions. Anal. Chem. **47:**916-918.

Lennox E. S. 1955. Transduction of linked genetic characters of the host bacteriophage P1. Virology **1**: 190-206.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265-275.

Lu, Z., E. A. DiBlasio-Smith, K. L. Grant, N. W. Warne, E. La Vallie, L. Collins-Racie, M. Follettie, W. Williamson, and J. McCoy. 1996. Histidine-patch thioredoxins; mutant forms of thioredoxin with metal chelating affinity that provide for convenient purifications of thioredoxin fusion proteins. J. Biol. Chem. **271:**5059-5065.

Metzler, D. 2001. Biochemistry. 2nd ed. Harcourt/Academic Press, San Diego/London.

Meyers, P. R., D. E. Rawlings, D. R. Woods, and G. G. Lindsey. 1993. Isolation and characterization of a cyanide dihydratase from *Bacillus pumilus* C1. J. Bacteriol. **175:** 6105-6112.

Podar, M., J. R. Eads, and H. Richardson. 2005. Evolution of a microbial nitrilase gene family: a comparative and environmental genomics study. BMC Evol. Biol. 5:42.

Poulton, J. E. 1988. Localization and catabolism of cyanogenic glycosides. In: Cyanide Compounds in Biology, CIBA Found Symp. **140**:67-81.

Rabilloud, T. 2000. Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods, Springer

Raybuck, S. A. 1992. Microbes and microbial enzymes for cyanide degradation. Biodegradation. **3**:3-18

Rhoads D. M., A. L. Ambach, C. R. Sweet, A. M. Lennon, G. S. Rauch and J. N. Siedow. 1998. Regulation of cyanide–resistant alternative oxidase of plant mitochondria. J. Biol. Chem. **273:** 30750-30756.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Nicklen, S., and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. (USA) **74**: 5463-5467

Sigdel, T.K., R. Cilliers, P. R. Gursahaney, and M. W. Crowder. 2004. Fractionations of soluble proteins in *Escherichia coli* using DEAE-, SP-, and phenyl sepharose chromatographies. J. Biomol.Tech. **15:**199-207.

Silva-Avalos, J., M. G. Richmond, O. Nagappan, and D. A. Kunz. 1990. Degradation of the metal-cyano complex tetracyanonickelate(II) by cyanide-utilizing bacterial isolates. Appl. Environ. Microbiol. **56:**3664-3670.

Skrowronski, B., and G. A. Strobel. 1969. Cyanide resistance and cyanide utilization by a strain of *Bacillus pumilus.* Can. J. Microbiol. **15:**93-98.

Solomonson, L. P. 1981. Cyanide as a metabolic inhibitor. p.11-28. In B. Vennesland et al. (ed), Cyanide in biology. Academic Press, Inc., New York.

Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450-490.

Studier, F. W., and B. A. Moffat. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189**:113- 130

Studier, **F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Meth. Enzymol. **185**: 60-89

Vergote, D., E. R. Macagno, M. Salzet, and . P.-E. Sautière. 2006. Proteome modifications of the medicinal leech nervous system under bacterial challenge. Proteomics. **6:** 4817-4825.

Wang, C. S., D. A. Kunz and B.J. Venables. 1996. Incorporation of molecular oxygen and water during enzymatic oxidation of cyanide by *Pseudomonas fluorescens* NCIMB 11764. Appl. Environ. Microbiol. **62:** 2195-2197.

Wang, P., D. E. Mathews and H. D. Van Etten. 1992. Purification and characterization of cyanide hydratase from the phytopathogenic fungus *Gleocercospora sorghi*. Arch. Biochem. Biophys. **298:** 569-575.

Ware, G. C., and H. A. Painter. 1955. Bacterial utilization of cyanide. Nature **175:**900.

Watanabe, A., K. Yano, K. Ikebukuro, and I. Karube. 1998. Cyanide hydrolysis in a cyanide-degrading bacterium *Pseudomonas stutzeri* AK61, by cyanidase. Microbiology **144**:1677-1682.

Westley, J. 1981. Cyanide and sulfane sulfur. P. 61-76. *In* B. Vennesland, E. E. Conn, C. J. Knowles. J. Westley, and F. Wissing (eds.), Cyanide in Biology. Academic Press, New York.