ACINETOBACTER ADP1 AS A SYSTEM FOR *IN VIVO* IDENTIFICATION OF BACTERIAL COPROPORPHYRINOGEN III OXIDASES

by

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(Under the Direction of Harry Dailey)

ABSTRACT

Current gene annotation methods based on sequence identity have greatly expanded the ability to search growing databases of genomic information. This system of identification falls short when novel genes are involved or when large protein families show homology across an array of protein functions. Such is the case with the bacterial oxygen-independent coproporphyrinogen III oxidase (CPO) HemN. Its association with the radical SAM protein superfamily has led to incorrect annotation of non-HemN proteins. Radical SAM family proteins are highly conserved and traditional sequence comparison proves near impossible for accurate identification of HemN proteins. An *in vivo* system was developed in the naturally transformable bacterium *Acinetobacter baylyi* ADP1. The wild-type CPO was deleted using basic PCR cloning techniques utilizing this organism's high competency for natural transformation and recombination. The resulting CPO deletion is presented as an *in vivo* system for identification of bacterial CPOs.

INDEX WORDS: Heme biosynthesis, Coproporphyrinogen III oxidase, *Acinetobacter* ADP1, Radical-SAM superfamily, Gene knockout

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Dedication

This is dedicated to my family, whose unwavering support has forever provided peace amidst the ebb and flow of becoming ones own.

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Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction and Literature Review

Overview

The primary objective of this project was to create a coproporphyrinogen III oxidase deletion in the Gram negative bacterium *Acinetobacter baylyi* ADP1. This heme auxotroph is to be used as a system for the identification of oxygen-independent coproporphyrinogen III oxidase function for genes currently lacking biochemical data to characterize them as such. The need for a system such as this is dictated by the drastic mis-annotation of genes belonging to the radical SAM protein superfamily. Oxygen-independent coproporphyrinogen III oxidase belongs to this family. The following introduction covers the heme biosynthetic pathway, coproporphyrinogen III oxidases, the annotation problem, and *A. baylyi* ADP1.

Heme

Heme is a multi-functional prosthetic group and regulatory molecule utilized in multiple capacities by oxidative, metabolic, and gene expression pathways. Metabolic uses included molecular oxygen transport, electron transfer, metabolism of oxygen and other diatomic gases, and oxidation-reduction reactions. Heme is the prosthetic group of hemoglobins, catalases, cytochromes, and peroxidases involved in aerobic metabolism. Additionally, heme proteins are involved in the sensing and synthesis of diatomic gases in both prokaryotes and eukaryotes [1]. *Mycobacterium tuberculosis* DevS is a heme-containing oxygen-sensing kinase associated with dormancy [2]. Another heme protein,

CooA, is a CO-sensing transcriptional activator in the Gram-negative bacteriaum *Rhodospirillum rubrum* [3]. In its regulatory capacity, there is evidence of heme mediation of gene expression at the level of transcription [4-7], translation [8], protein stability [9, 10], protein targeting [11], and differentiation [12]. In prokaryotes, cytochromes are the most abundant heme proteins and are essential for respiration. Oxidative respiration under oxygen-deficient conditions require the use of other oxidants such as nitrate or sulfate. As in aerobic respiration cytochromes mediate electron transfer as well as the ultimate reduction of the terminal electron receptor [13, 14]. Hemes are found highly abundant in the bacteria and archaea and demands are met primarily by biosynthesis rather than acquisition from the environment [15].

Tetrapyrrole biosynthesis

The C-4 and C-5 pathways

Hemes are one member of the tetrapyrrole family of organic compounds. These include linear bilins, corrins, and metalloporphyrins including chlorophylls and usually act as essential components for a cell's metabolism. Two biosynthetic pathways exist for the first universal tetrapyrrole precursor, 5-aminolevulinic acid (ALA) (Figure 1). These two pathways are marked by their respective starting compounds; glycine and succinyl-CoA (C-4 pathway) or glutamyl tRNA (C-5 pathway). Within bacteria only members of the α-proteobacteria utilize the C-4 pathway, which is a single step condensation of glycine and succinyl-CoA catalyzed by ALA synthase (ALAS) to form ALA and an eliminated carbon dioxide. This pathway is also found in non-photosynthetic eukaryotes including fungi and animals. All other bacteria, as well as archaea and

plants, use the C-5 pathway for synthesis of ALA, which gets its name from the 5-carbon skeleton of glutamate. Two committed enzymes, glutamyl-tRNA reductase and glutamate 1-semialdehyde (GSA) aminotransferase, are involved in the three step synthesis of ALA via the C-5 pathway (Figure 1). The first step is non-committed as glutamyl-tRNA synthetase also charges $tRNA^{Glu}$ with glutamate during protein synthesis (Schon *et al.*, 1986; Schneegurt and Beale, 1988). Glutamyl-$tRNA^{Glu}$ is then reduced to GSA by glutamyl-tRNA reductase using NADPH, and finally ALA is produced from GSA via transamination **[13]**.

ALA Synthase (hemA)

Bacterial ALAS, encoded by the *hemA* gene, shares 49% sequence identity with human ALAS and has been most studied in *Rhodobacter sphaeroides* and in the rhizobia. *R. sphaeroides* has been shown to contain two *hemA* homologues, designated *hemA* and *hemT* **[16]**. The designation *hemA* is also used in reference to the gene encoding bacterial glutamyl-tRNA reductase in spite of being a dissimilar protein catalyzing a different reaction than bacterial ALAS. It has been suggested the C-5 associated gene be redesignated as *gtrA* which seems to be an ideal resolution to this issue **[13, 14]**.

Uro'gen III synthesis

The three enzymatic steps following ALA synthesis are highly conserved in the biosynthesis of all tetrapyrroles and catalyze the formation of uroporphyrinogen III (uro'gen III), the final common precursor in tetrapyrrole formation (Figure 2).

ALA dehydratase (hemB)

ALA dehydratase (ALAD), which is also referred to as porphobilinogen (PBG) synthase (PBGS), is encoded in bacteria by *hemB,* is found in all organisms that synthesize tetrapyrroles. The enzyme is a homo-octamer composed of four dimers that catalyzes the condensation of two ALA molecules to form PBG. It requires one divalent cation per subunit that is essential for activity. While the animal and yeast proteins are zinc-binding, and the plant enzyme requires Mg^{2+}, bacterial ALADs have diverse metal requirements. In addition to proteins binding monovalent ions, enzymes utilizing Zn^{2+}, Mg^{2+}, or both having been characterized **[17]**. Human ALAD has been shown to exist in quaternary structure assemblies known as “morpheeins”, which are functionally distinct from each other. High-activity octamers and low-activity hexamers have been observed **[18]**. It has not been determined if a similar phenomena exists for bacterial ALAD.

PBG deaminase (hemC)

PBG deaminase (PBDG), also called hydroxymethylbilane synthase, is encoded by *hemC* and catalyzes the formation of the linear tetrapyrrole 1-hydroxymethylbilane (preuroporphyrin I) by the head to tail condensation and concomitant deaminization of four molecules of PBG. PBGD was the first tetrapyrrole pathway enzyme to have its structure determined **[19]**. The enzyme is a homotetramer that synthesizes its own dipyrrole cofactor. This dipyrromethane cofactor is a PBG dimer covalently linked to a cysteine residue **[20-22]**. Available genetic data indicates the protein sequence is highly conserved and the enzyme is found in all tetrapyrrole-synthesizing organisms.

Uropophyrinogen III synthase (hemD)

The linear tetrapyrrole hydroxymethylbilane is an unstable intermediate and is readily cyclized nonezymatically to form uroporphyrinogen I. However this symmetric isomer of uro'gen III is not physiologically useful. Uro'gen III synthase (UroS), encoded by *hemD*, catalyzes inversion of the D pyrrole ring as it cyclizes hydroxymethylbilane into uroporphyrinogen III, the first macrocycle in the pathway and the final common intermediate for all tetrapyrroles. UroS is a monomer with a molecular mass around 30,000. Little sequence similarity has been seen across different organisms. In bacteria which contain both a *hemC* and *hemD* gene, they are often found clustered together, leading to coordinated expression of both genes. However, examination of available prokaryotic genomes indicates a significant number of organisms do not possess an identifiable *hemD* gene. In certain organisms this absence is the result of *hemD* being fused with *cobA* **[23]**. The *cobA* gene encodes uroporphyrinogen III methyltransferase. Its activity is not involved in protoporphyrin biosynthesis but rather is associated with cobalamin and siroheme synthesis. The gene fusion shows both CobA and HemD activity, and will rescue an *E. coli hemD* mutant. Gene fusions with *hemC*, such as that in *Leptospira interrogans,* have also been found. These fusions are reported to posses HemD activity. Even when identified gene fusions are taken into consideration there exists a large group of organisms, across multiple taxonomic groups, without an identifiable *hemD*.

Protoporphyrin IX Synthesis

The four final steps of heme biosynthesis culminate with insertion of ferrous iron into proporphyrin IX forming protoheme, the end product of the heme biosynthetic pathway (Figure 2).

Uroporphyrinogen III decarboxylase (hemE)

The decarboxylation of uro'gen III to coproporphyrinogen III (copro'gen III) represents the first committed step of protoheme IX synthesis. It is catalyzed by the enzyme uro'gen III decarboxylase (UroD) which is encoded by the bacterial gene *hemE*. This enzyme, which possesses no cofactors, decarboxylates the four acetate side chains of uro'gen III to methyl groups and releases 4 molecules of CO_2 per molecule of uro'gen III. Decarboxylation under physiological conditions occurs in a specific sequence, starting with the acetate on ring D, followed by A, B, and then C. At high substrate concentrations the sequence has been shown to be random **[24]**. The structure of UroD is known and reveals the protein to be a homodimer with an active site in each monomer **[25]**. This evidence supports previous suggestions that all decarboxylations occurred at a single active site **[26]**. HemE is highly conserved in bacteria and is often present in organisms known to synthesize protoporphyrin IX clustered with other genes associated with protoheme IX biosynthesis.

Coproporphyrinogen III oxidase (hemF, hemN)

The antepenultimate reaction in protoheme IX biosynthesis is catalyzed by the enzyme copro'gen III oxidase (CPO). This step is characterized by the sequential

oxidative decarboxylation of propionate side chains at positions 2 and 4 to form vinyl groups, and results in the formation of dicarboxylate protoporphyrinogen IX (proto'gen IX). The monovinyl intermediate harderoporphyrinogen (hardero'gen) results from the first decarboxylation **[27]**. Two distinct enzymes have been characterized which catalyze this reaction. While the substrates and products of these two enzymes are identical, the proteins themselves are dissimilar in their primary sequences. An oxygen-dependent enzyme, encoded in bacteria by *hemF*, is conserved among eukaryotic organisms and is also found in numerous bacteria. Bacterial HemF is a membrane-associated homodimer which has 60-70% similarity with plant and animal CPOs and is approximately 300 amino acids in length. Human CPO is well characterized and has been shown to contain no cofactors or metals **[28]**. The association of manganese with *E. coli* hemF activity has been documented though a clear definition of its role has yet to be determined **[29]**.

An oxygen-independent form of the enzyme also exists in some prokaryotes and is encoded by the *hemN* gene. The HemN protein contains a [4Fe-4S] cluster and requires two molecules of S-adenosylmethionine (SAM) to catalyze the reaction **[30]**. HemN has been classified as a member of a large class of SAM-dependent or 'radical SAM' enzymes **[31]** and the requirement of two SAM molecules for catalysis has been previously described for other radical SAM enzymes **[32, 33]**. *Escherichia coli* HemN was the first radical SAM protein for which the structure was determined and the proposed catalytic mechanism of HemN is based on this structural, as well as kinetic data **[34]**. Both *hemF* and *hemN* are found together in certain organisms, one of the best documented of these being *E. coli* **[29, 35]**. Recently, both HemF and HemN have

been biochemically identified in the cyanobacterium *Synechocystis* sp. PCC 6803 **[36]**. Additionally, the presence of two genes encoding oxygen-independent CPOs has been reported in multiple organisms. These homologs have been labeled *hemN* and *hemZ* in some reports **[37]** and *hemN1* and *hemN2* by others **[38]**. For organisms with two *hemN* homologues, evidence indicates often only one of the two may be an active protein. In *Bradyrhizobium japonicum*, *hemN2* was shown to be required for anaerobic growth and symbiosis with soybean, while a *hemN1* mutant had no phenotype **[38]**. In addition, only the *hemN2* gene was shown to complement a *Salmonella typhimurium hemF hemN* double knockout strain. Similar findings have been described for the two *hemN*-like genes in *Synechocystis* **[36]**. Both *hemN* homologues (*hemN*, *hemZ*) in *Bacillus subtilis* have been shown to have CPO activity **[37, 39]**. The *hemN* gene is clustered with other microaerobic respiration genes in *R. sphaeroides* **[40]**, *Mesorhizobium loti* **[41]**, and *Paracoccus denitrificans* **[42]**. The dissimilar HemF and HemN enzymes have been shown to compliment each other in mutant phenotypes. In *S. typhimurium*, which contains both genes, no growth impairment was observed in a *hemF* mutant while a *hemN* mutant was shown to be heme auxotrophic only under anaerobic growth **[43]**. Additionally, a *hemF hemN* double mutant was complimented by HemF only under aerobic conditions, whereas *hemN* will confer normal growth aerobically or anaerobically. These studies highlight the classification of these two proteins as oxygen-dependent or independent. Additionally, they show that while HemN can function in an aerobic environment, it does not require oxygen for activity nor does oxygen appear to inhibit its ability to carry out catalysis.

The presence of both types of CPO in organisms seems to be a mechanism for dealing with fluctuating environmental oxygen levels. Gene transcription studies have shown significant up-regulation of oxygen-independent CPO transcription under anaerobic conditions **[35, 36]**. Other findings have shown HemN transcription at roughly equivalent levels under aerobic and anaerobic conditions **[39]**. With regards to oxygen-dependent CPO expression, some studies have shown constitutive expression of HemF under all oxygen levels **[36]** while others have observed oxygen dependent regulation **[44]**. While oxygen-tension transcription regulation is an active indication of response to environmental oxygen levels, the presence and expression of both forms of CPO stands as evidence of microbial adaptation to dynamic micro-environments. Further attention will be given to both forms of CPO later in this review.

Protoporphyrinogen IX oxidase (hemY, hemG)

The six electron oxidation of proto'gen IX to protoporphyrin IX represents the penultimate step in heme biosynthesis. Proto'gen IX oxidase (PPO) is the enzyme responsible and like CPO, oxygen-dependent and oxygen-independent forms exist. The bacterial proteins are encoded by the *hemY* (oxygen-dependent) and *hemG* (oxygen-independent) genes. In contrast to the CPOs, HemY and HemG are not found together in a given cell. The oxygen-dependent form is found in all heme synthesizing eukaryotes as well as most Gram-positive bacteria, but in very few Gram-negative bacteria. Currently, no archaea have been found to contain this protein. HemY is a homodimer containing FAD and can utilize O_2 as its terminal electron receptor. Bacterial HemY is part of an FAD-containing amine oxidase superfamily and is homologous to the

eukaryotic oxygen-dependent PPO, though equal sequence identity is observed between HemY and other amine oxidases **[45]**.

The oxygen-independent PPO encoded by the *hemG* gene has only recently been characterized as having PPO activity in *E. coli* **[46]**. This evidence highlights a menaquinone-depdendent enzyme approximately 22 kD of the long chain flavodoxin family unique to the γ-proteobacteria. This accounted for, known PPOs are still lacking for any *Archaea* and roughly 50% of currently sequenced bacterial genomes. The activity of these 'missing' PPOs may potentially be mediated by multiple proteins as is described in *Desulfovibrio gigas* where three different proteins are shown to carry out the reaction **[47]**, but an unclassified PPO protein is likely responsible.

Ferrochelatase (hemH)

Insertion of ferrous iron into protoporphyrin IX, yielding protoheme, is the terminal step in heme biosynthesis and is catalyzed by a metal chelatase. This ferrochelatase is a Class II chelatase and is encoded by the *hemH* gene in prokaryotes. *In vitro* some divalent ions such as Co^{2+} and Zn^{2+} can server as a substrate, while others such as Mn^{2+}, Cd^{2+}, and Pb^{2+} are competitive inhibitors **[48]**. Across prokaryotes and eukaryotes ferrochelatase is conserved though few amino acid residues are conserved universally. Animal ferrochelatases are [2Fe-2S] proteins **[49]** with 3 of the coordinating cysteines in the C-terminus **[50]**. In plant ferrochelatases these cysteines are missing while in many bacteria the entire C-terminus is absent. Bacterial proteins with C-terminal extensions containing cysteine clusters have been identified but these are distinct from the C-terminal regions of animal ferrochelatases **[48]**. Eukaryotic ferrochelatases are membrane-associated homodimers while variation is seen in prokaryotes. Gram-

positive bacteria have soluble monomeric HemH while Gram-negative species have membrane-associated homodimeric HemH. Iron-sulfur clusters are unique to the animal, *Actinobacterial*, and some Gram-negative versions of the protein.

The Annotation Problem

Based on currently annotated prokaryotic genomes, one can elucidate the number of identified enzymes for each step of heme biosynthesis. For the majority of the pathway, genes are accounted for in most available genomes. This is primarily through direct annotation of the responsible gene, or through identified genetic alternatives such as the *hemD/cobA* fusion described previously. As outlined above, identification of valid PPOs is still lacking in about 50% available bacterial genomes and all archaea placing PPO at the head of the list of 'missing' prokaryotic heme synthesis enzymes. Behind PPO, biochemically validated CPOs are yet to be identified in the vast majority of archaea and in many bacteria as well. A simple search of the Entrez gene database yields 1683 entries for *hemN* (or annotated as an oxygen-independent coproporphyrinogen-III oxidase) in prokaryotic genomes and the vast majority of these genes have been annotated as 'CPO-like radical SAM enzymes' without supporting biochemical data. Currently, there are no apparent issues regarding *hemF* annotation, the same cannot be said of *hemN*. While continually increasing numbers of annotated bacterial genomes have made genome-wide data mining for metabolic pathway genes an effective method of identifying new enzymes, this technique falls short when novel or unique genes are involved or when the gene target shares significant similarity with genes indifferent to the targeted processes. The latter of these issues is particularly prevalent in protein superfamilies, as has been recently highlighted **[51]**. High levels of

sequence identity in many protein superfamilies leads to these annotation issues which come into play when considering bacterial HemN and its paralogs in the radical SAM superfamily.

Radical SAM enzymes

Radical SAMS were first classified in 1970 with the characterization of *Clostridium subterminale* lysine 2,3-aminomutase (LAM) **[52, 53]**. Under current classification, the radical SAM superfamily is divided into two main subfamilies **[54]**. Subfamily 1 contains proteins for which the reactions involve glycyl radical intermediates and require an activase, for enzymes in subfamily 2 the reaction proceeds with a direct hydrogen transfer between the substrate and the 5'-deoxyadenosyl radical (Figure 3). HemN is a member of this second subfamily. The first steps of the reaction begin with iron-sulfur cluster reduction and SAM cleavage which result in the formation of a 5'-deoxyadenosyl radical. Recent counts have the radical SAM superfamily covering more than 2800 proteins and more than 40 distinct biochemical transformations **[55]**. Most members have not been biochemically characterized.

All radical SAM enzymes are related by the cysteine motif CxxxCxxC which nucleates the [4Fe-4S] cluster of each **[56]**. In addition to the cysteine motif and the necessity of SAM as a cofactor for radical generation, all radical SAM enzymes have a conserved glycine-rich sequence motif which is believed to be involved in SAM binding **[57]**. These similarities within the family have lead to SAM family proteins paralogous to HemN being erroneously annotated as 'potential oxygen-independent CPOs' without supporting biological or biochemical data. This problem is exemplified by the *Vibrio*

cholera protein HutW, a HemN paralog which has been shown to lack oxygen-independent CPO activity. HutW has been annotated as a CPO in genomic databases and previous work indicates it is associated with exogenous heme uptake **[4]**. HutW and all of its orthologs are shown to possess a CxxxCxxC motif, glycine motif, as well as other conserved residues found to be essential for iron-sulfur cluster formation and CPO function **[58]**. As such, it is clear that while these traits are necessary in a functional oxygen-independent CPO, they alone are inadequate for identification of radical SAM-based CPOs. Additionally, this example demonstrates that accurate homology-based analysis of this family is near impossible. This issue may be avoided by careful analysis of genomic data. By searching annotated genomes for HemN orthologs co-localized with known genes involved with heme transport the current group of CPO-like radical SAM enzymes can be divided into four groups: 1) bona fide CPO enzymes, 2) radical SAM subfamily 1 proteins found in Gram negative bacteria, 3) radical SAM subfamily 2 proteins found in Gram positive bacteria, and 4) HutW-like radical SAM family proteins. Using this logic and current information on heme pathways there are roughly 660 bacteria capable of heme biosynthesis. Of these a loose estimate of 440 contain 'real-hemN' enzymes (Svetlana Gerdes personal communication, SEED).

CPO-Mechanisms

Mechanistic differences between the two types of CPO enzymes parallels their overall dissimilarity. Oxygen-dependent CPO catalysis proceeds through a side-chain mediated decarboxylation of ring A, followed by ring B, utilizing molecular oxygen as electron acceptor and resulting in release of H_2O_2 and CO_2. While the exact catalytic

sequence of the O_2-dependent enzyme remains unclear, two mechanisms have been discussed for this oxidative decarboxylation. Both are based on the initial base-catalyzed deprotonation of the pyrrole NH, giving an azacyclopentadienyl anion (Figure 4). In the first proposed mechanism, the reaction proceeds through a one-electron oxidation of the substrate by dioxygen, followed by hydrogen abstraction from the propionate side chain by the resulting superoxide anion. This is followed by decarboxylation using the electron-deficient pyrrole as an electron sink **[59]**. In the second mechanism the deprotonated pyrrole ring would then be able to attack dioxygen forming a two hydrogen-pyrrole peroxide anion which could abstract a proton from the propionate side chain, thus allowing decarboxylation **[60]**. While functional studies have indicated this second mechanism may be more energetically favorable **[61]**, concrete evidence is lacking to confirm this to as the primary catalytic mechanism of oxygen-dependent CPO. It is also feasible to consider a combination of the two mechanisms though this has yet to be pursued. In both routes described above, the product will be the monovinyl harderoporphyrinogen intermediate which will undergo a second catalytic cycle resulting in the final protoporphyrinogen product of the CPO enzyme. Recent evidence indicates oxygen-dependent CPO can metabolize a variety of hardero'gen isomers **[62]**. This seems only to prove CPO may be less selective than originally believed and does not appear to have implications on the previously described catalytic mechanisms.

While the entire mechanism of of bacterial HemN has not been fully confirmed the enzyme's association with the radical SAM family provides well documented primary catalytic steps on which to formulate a possible catalytic sequence. The proposed

mechanism begins with the formation a 5'-deoxyadenosyl radical from homolytic cleavage of SAM as described above (Figure 3). This radical then abstracts a hydrogen from the β-C of the propionate side chain of the substrate to yield a substrate radical and 5'-deoxyadenosine (Figure 3). The formation of this radical has been characterized **[63]**. The final step includes vinyl group formation and CO_2 release and requires an electron acceptor for the electron of the substrate radical.

HemN Homologues

While classification of genes through functional context is a logical solution to the annotation problem, defining 'functional context' is vital to its success. It has already been detailed that many organisms contain multiple homologues of *hemN* and that often only one of the two is shown to have CPO activity. In Gram-negative organisms, homologues belonging to radical SAM subfamily 1 do not co-localize with other genes of heme biosynthesis. Members of this subfamily are also often found in organisms such as *Haemophilus influenzae*, *Bartonella*, *Buchnera*, and *Thermotogaceae* which lack tetrapyrrole biosynthesis. Coupling of these genes to nucleoside 5-triosphatase RdgB and sometimes ribonuclease PH may indicate their involvement in oxidative stress response pathways instead of heme biosynthesis.

In Gram-positive organisms the picture is less clear. The gene for *hemF* is lacking in all Gram-positive organisms with genomes currently sequenced, but at least one copy of *hemN* or *hemZ* is found. As previously outlined, *B. subtilis* has two functional CPOs in *hemN* and *hemZ*, both of which will compliment a *S. typhimurium hemN hemF* double mutant. Orthologs of the *B. subtilis hemN* across the Gram-positives do not co-localize with heme bio-synthesis genes. Rather, they are found with

heat shock genes and some genes potentially involved with ribosomal stress responses. Orthologs are also found in organisms such as *Streptococcus, Clostridia,* and *Enterococcus,* which lack other genes associated with heme-biosynthesis. This presents the question of whether their orthologs in heme-biosynthesis organisms will have functional CPO activity. Testing members of each of these ortholog groups for CPO activity should prove instructive in clarifying these discrepancies.

With current annotation methods failing to accurately identify uncharacterized CPO enzymes more biological data is needed to expand the library of confirmed CPOs and better establish a 'CPO footprint' on which to build further computational data. It is important to test enzymatic function of HemN orthologs from organisms with functional heme biosynthesis as well as organisms without it. Based on current information, it would be expected that orthologs from organisms lacking heme biosynthesis would not show CPO activity, though this possibility cannot be ruled out due to the possibility of paralogous gene displacement. While over-expression and purification of 'possible CPOs' could prove effective in this matter, using gene-rescue of a CPO knockout seems to be a more efficient way to confirm CPO activity and will allow rapid screening of multiple potential enzymes.

Acinetobacter

Acinetobacter baylyi ADP1 is a Gram-negative soil bacterium closely related to members of *Pseudomonas aeruginosa and Pseudomonas putidas* as determined by comparison of 16S RNA genes. ADP1 has a relatively compact genome of 3.7 Mb, <60% of the length of the *Pseudomonas* counterparts, and an average G+C content of

40.3%; versus 62% in *P. aeruginosa* [64]. *Acinetobacter* spp. are prevalent in nature and can be obtained from water, soil, and living organisms. These nutritionally versatile chemotrophic bacteria are strictly aerobic and tend to be paired non-motile [64]. Clinical interest in this genus, particularly regarding *Acinetobacter baumannii* and its close relatives, has increased as their role in multi-drug resistant hospital infections has been documented [65, 66]. More recently, *Acinetobacter baylyi* has gained significant research interest for its potential as a model organism. *A. baylyi* displays an extraordinary competence for natural transformation and natural recombination, on the order of 10-100 times as competent as calcium chloride treated *E. coli* [67], with strain ADP1 and its closest relatives showing the highest levels of competency among *A. baylyi* strains [68]. ADP1 was originally isolated as a derivative of *A. baylyi* strain BD4 [69]. Strain BD4 forms mucoid colonies which make it difficult for laboratory use. This resulted in utilization of a microencapsulated BD4 mutant originally designated 'strain BD413' and now known as ADP1 [68]. Genetic manipulation of *A. baylyi* ADP1 is as simple as direct addition of linear or plasmid DNA to log-phase cultures and was first indicated 40 years ago [70]. The potential of *A. baylyi* as a model organism is further complemented by its catabolic diversity and nutritional versatility [71, 72]. This combination of characteristics results in *A. baylyi* ADP1 being an ideal organism for manipulative genetic investigation of metabolic and catabolic pathways.

Acinetobacter ADP1 Heme Synthesis

A. baylyi ADP1 synthesizes ALA by the C-5 pathway and possesses the *gtrA* gene (ACIAD2900). A simple search of the complete ADP1 genome produces genes for

all steps of heme biosynthesis (Table 1) with the exception of PPO. However, recent findings suggest *cog1981* (ACIAD0878) may have PPO activity. An ADP1 *cog1981* mutant was complimented by HemG (unpublished data). Note: *cog1981* has been designated *hemJ*. ADP1 has only a single copy of the oxygen-dependent *hemF* gene. There is a putative *hemN*-like CPO currently annotated but this gene lacks CPO activity and is unable to rescue a hemF knockout (this study). The presence of only HemF seems appropriate considering the organism is strictly aerobic and is not known to operate under low-oxygen conditions.

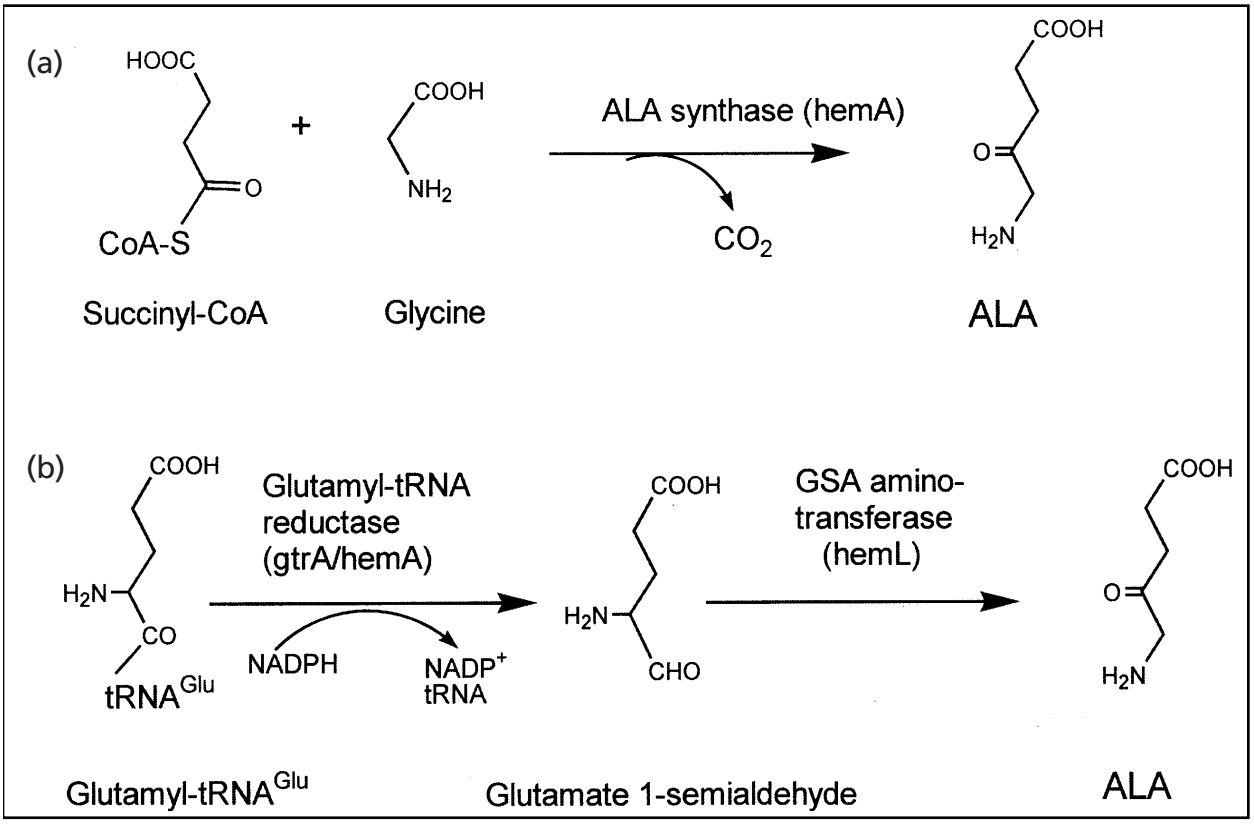

Figure 1: Both routes of ALA synthesis. (a) The C4 pathway utilizes ALA synthase (*hemA)* and (b) the C5 pathway utilizes glutamyl-tRNA reductase (*gtrA*). The formation of Glutamyl-tRNA is not shown and is catalyzed by glutamyl-tRNA synthetase [14].

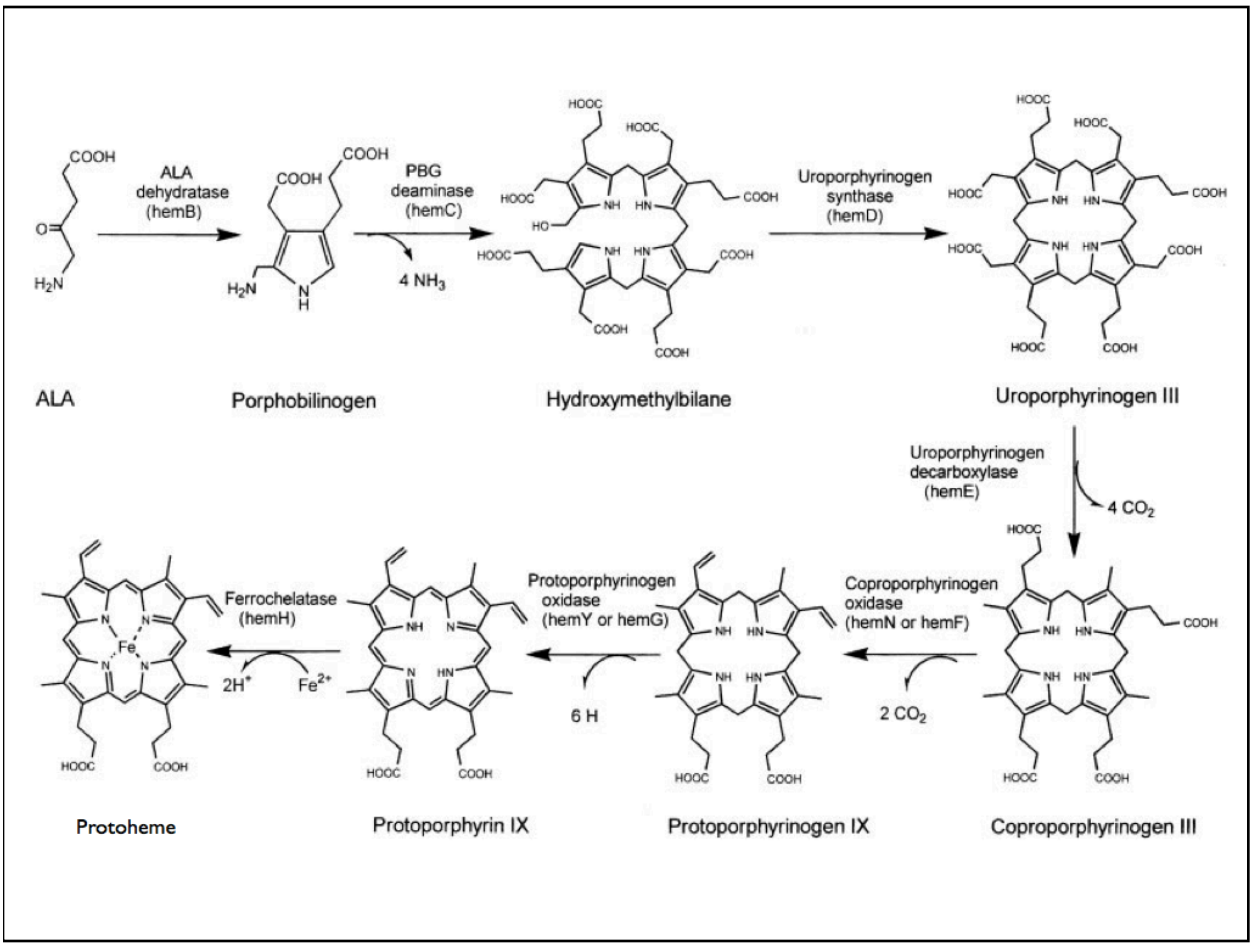

Figure 2: The steps of heme biosynthesis from ALA to protoheme. The enzymes associated with each step are noted along with the genes that encode them [14].

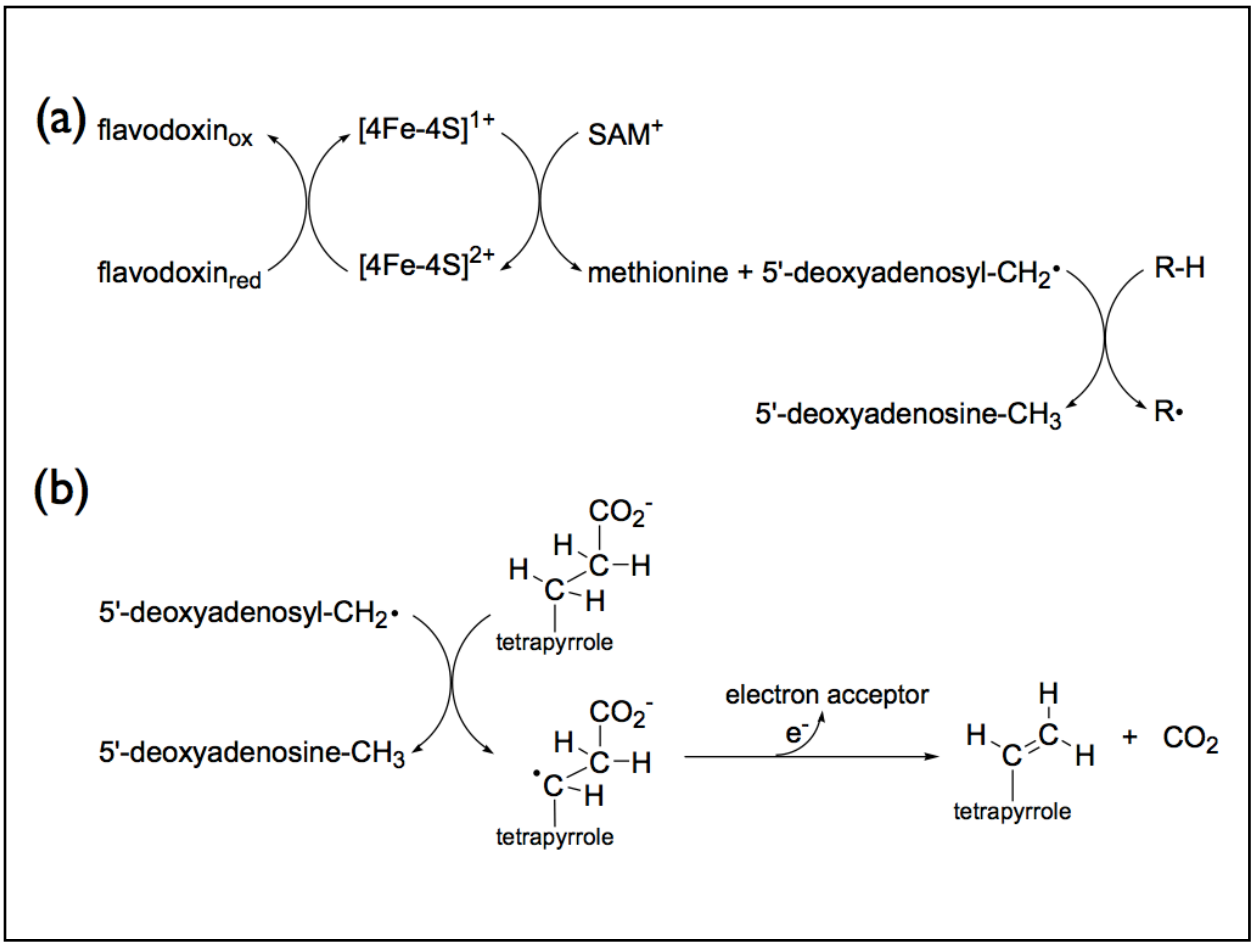

Figure 3: Proposed mechanism for Radical SAM enzymes. (a) The initial reaction steps are common to all radical SAM enzymes and include the reduction of the iron-sulfur cluster, SAM cleavage and formation of a 5'-deoxyadenosyl radical as well as H-atom abstraction from the substrate. (b) The proposed mechanism for HemN involves H-atom abstraction from the propionate side chain. Product formation is preceded by release of CO_2 and uptake of the remaining electron by an unknown electron acceptor [73].

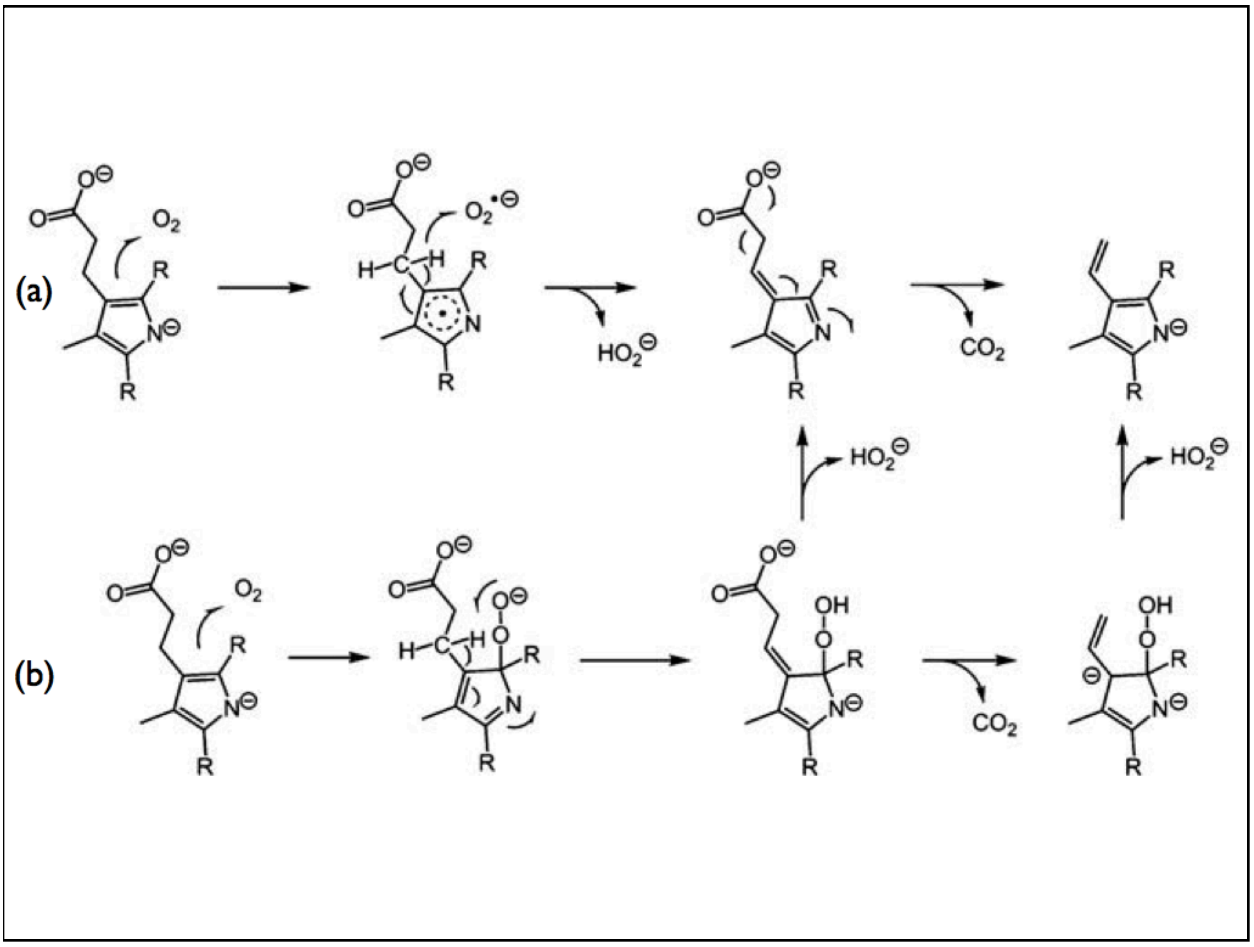

Figure 4: Two proposed reaction mechanisms for oxygen-dependent CPO. (a) One-electron oxidation of the substrate by dioxygen results in a superoxide anion and a pyrrole radical. Hydrogen-abstraction from the propionate side chain by the superoxide anion is followed by decarboxylation. (b) The deprotonated pyrrole substrate attacks dioxygen forming a 2H-pyrrole peroxide anion which abstracts a proton from the propionate side chain allowing for decarboxylation [74].

Table 1: Heme biosynthesis genes in *Acinetobacter* ADP1.

Protein	***Gene***	***Gene ID***
Glutamyl-tRNA reductase	*hemA (gtrA)*	ACIAD2900
ALA dehydratase	*hemB*	ACIAD0923
PBG deaminase	*hemC*	ACIAD0286
Uro'gen III synthase	*hemD*	ACIAD0287
Uro'gen III decarboxylase	*hemE*	ACIAD2474
Copro'gen III oxidase	*hemF*	ACIAD3250
Proto'gen IX oxidase	*cog1981* (*hemJ*)	ACIAD0878
Ferrochelatase	*hemH*	ACIAD3255

Chapter 2: A Bacterial CPO Knockout System

Overview

The following describes the methodology behind the development of a CPO deletion/substitution system for characterization of CPO-like genes. Note: Strain and vector names used in this thesis are unconventional and have been replaced with ACN strain numbers and pBac vector IDs.

Background

The need for biochemical data to supplement genomic information has been described in detail along with the potential merits of developing a knockout system with the flexibility to test genes from multiple bacterial sources. *E. coli* is our most well characterized prokaryotic gene expression system and is supported by an abundance of cloning and expression data. Additionally, the heme pathway in *E. coli* is well documented and it has well characterized *hemN* and *hemF* genes. However, past efforts to generate a *hemN*/*hemF* double mutant in *E. coli* have proven unsuccessful, eliminating *E. coli* as a possible system. Previous studies have used a *hemN*/*hemF* double knockout in *S. typhimurium* to characterize CPO enzymes [35, 39]. This system was a viable option but was unavailable for this study. Because both types of CPO have been shown to be active under aerobic conditions, an effective system would require the aforementioned double mutant with loss of function of both CPO genes. Creation of a new system in an aerobic Gram- bacteria containing a single oxygen-dependent

hemF provided the best chance for long-term application of the system. *A. baylyi* ADP1 meets these criteria. It does contain a putative *hemN* gene, however this gene was not believed to provide CPO activity and will be discussed further later in this study. ADP1 also displays a very high level of natural competency and is capable of natural recombination of homologous DNA. With the entire heme biosynthetic pathway accounted for ADP1 is an ideal candidate for the study of heme biosynthesis. Additionally, previous research indicating ADP1 expresses heterologous genes effectively [67] further presents this organism as an ideal system for a CPO knockout to help clarify the extensive issues previously mentioned regarding classification of associated oxygen-independent CPO genes.

Natural competency for both plasmid DNA and linear segments and a high efficiency in natural recombination of homologous DNA means both plasmid and linear DNA methods can be used to introduce heterologous genes into the ADP1 genome. Linear DNA constructs proved highly effective for deletion of the native CPO gene (Chapter 3) and this seems to be the most efficient method for accomplishing a clean gene deletion in ADP1. Developing an effective method of gene replacement presents more options.

Methodology

The Plasmid Route

Complementation of the *A. baylyi hemF* deletion was intended to be done using a plasmid construct as a carrier and expression vector for the genes tested. This presented as an ideal choice for the system for several reasons. Having a multi-copy number plasmid in the cell would ensure sufficient expression of the target protein to

avoid false negatives in complementation. Additionally, it would allow for antibiotic selection of cells containing the plasmid. The ADP1 strain has no native antibiotic resistance cassettes (though antibiotic resistance associated genes are present) and contamination becomes an issue if strict aseptic technique is not followed. Secondarily, having the plasmid confer resistance would allow for transformation into a knockout strain with a clean *hemF* deletion (Chapter 3). The plasmid chosen to do this is a modified version of the plasmid pBBR1MCS which has been used to complement a PPO knockout in *A. baylyi* (unpublished data). This vector confers chloramphenicol resistance and has had a ribosomal binding site cloned upstream of the multi-cloning site. Selection on chloramphenicol (20ug/ml) was to be followed by testing for heme biosynthesis complementation without exogenous heme present.

Unfortunately this approach was ineffective and we were unable to duplicate the previous success using this plasmid for complementation. The reasons for these mixed results are largely unclear. Chloramphenicol has not been widely used in *A. baylyi* and may not work at the same levels used in *E. coli*. However most common antibiotic resistance cassettes used in *E. coli* systems have been shown to be effective in ADP1 (ampicillan is not a favorable choice in ADP1 as the genome contains several β-lactam resistance factors). Furthermore, the combined effect of the gene knockout and resulting molecular response to scavenge exogenous heme, along with the expression of a heterologous gene on the plasmid, may have introduced complications in the uptake and utilization of the plasmid. Work using plasmid transformation in *A. baylyi* is far less common than transformation and recombination with linear DNA. There are no indications ADP1 cannot use certain expression vectors optimized for *E. coli*, but further

work needs to be done to optimize these existing expression systems in order to take advantage of the natural competency of *A. baylyi* ADP1.

The Recombination Route

The majority of published work on *A. baylyi* genetic modification has used transformation of linear DNA to manipulate the genome. The propensity of this species for uptake and homology-based recombination of linear DNA fragments has been studied [75]. This method is quite simple and can be adapted to fit most desired genetic outcomes. Recombination is dependent on homology with genomic DNA and simply requires flanking regions be cloned from the desired region of the *A. baylyi* genome (Figure 5). A downside to recombinant techniques can be in selection for recombinant cells if a clean deletion of the gene is desired. If recombinant cells have a selective resistance over non-recombinant cells such as with the *sacB*-Km^r cassette used in this study, then antibiotic selection for recombinants is no different than standards used for plasmid transformations. For this study, *A. baylyi hemF* was substituted with the 3.6kb *sacB*-Km^r cassette used in previous work in ADP1 [76]. This allows not only for antibiotic selection on kanamycin, but selection against the levansucrase encoded by the *B. subtilis sacB* gene when the cassette is crossed out by another recombination. Expression of the *sacB* gene has been shown to be lethal when sucrose is present. It is important to note that while selection against non-recombinants is possible, the Km^r cassette is crossed out along with the *sacB* gene and aseptic technique is essential to prevent contamination. An additional complication was encountered using sucrose to select against the *sacB* gene. Previous studies using this selective method employed 5-6% sucrose for selection against expression of the *sacB* gene, and this was used successfully in this study as well. However this method is optimal when selecting

against a low-cell count that is well dispersed on a selective plate such as in plating a transformation done in liquid culture or plating of a single colony like those in previous studies. The effectiveness of sucrose selectivity under these parameters was duplicated and observed for this study as well. However, the plate-based transformation methods we used (Chapter 3) result in a high quantity of cells being introduced onto the selective plate (by plating cell growth with a sterile loop). While efforts can be made to limit the amount of cells spread onto each plate, this method inevitably results in regions of high cell density on the selective sucrose media. It was found this resulted in growth of non-recombinant colonies still containing the *sacB* gene (Figure 6). This was observed previously [67, 76] and it was found that up to 70% of colonies from sucrose counter-selection plates were spontaneous loss-of-function mutants. It appears we have confirmed this observation, though no statistical analysis of our findings was done. With this in mind, it would seem the ideal solution would be the introduction of an alternative system of selectivity. This would also prove beneficial as it would reduce the possibility of contamination after crossing out the kanamycin resistance gene.

In spite of these setbacks the *hemF* deletion was successfully rescued with the functional *A. baylyi hemF*, confirming that recombinant methods are effective in complementing the gene knockout system. The *hemF* deletion phenotype alone has helped clarify annotation on a class of CPO-like radical-SAM enzymes of which many were mis-annotated as oxygen-independent CPOs (Chapter 3).

The Big Picture

The implications of this study can be measured both in the immediate data obtained and the possibility of future applicability of the system developed to test other possible CPO enzymes. Additionally, the methodology used adds to the limited but

growing bank of information on genetic manipulation in *A. baylyi* ADP1. Continued efforts in this organism should help prevent issues such as those encountered in this study. This study is a testament to the versatility of ADP1 as a system for genetic modification and expression of heterologous genes.

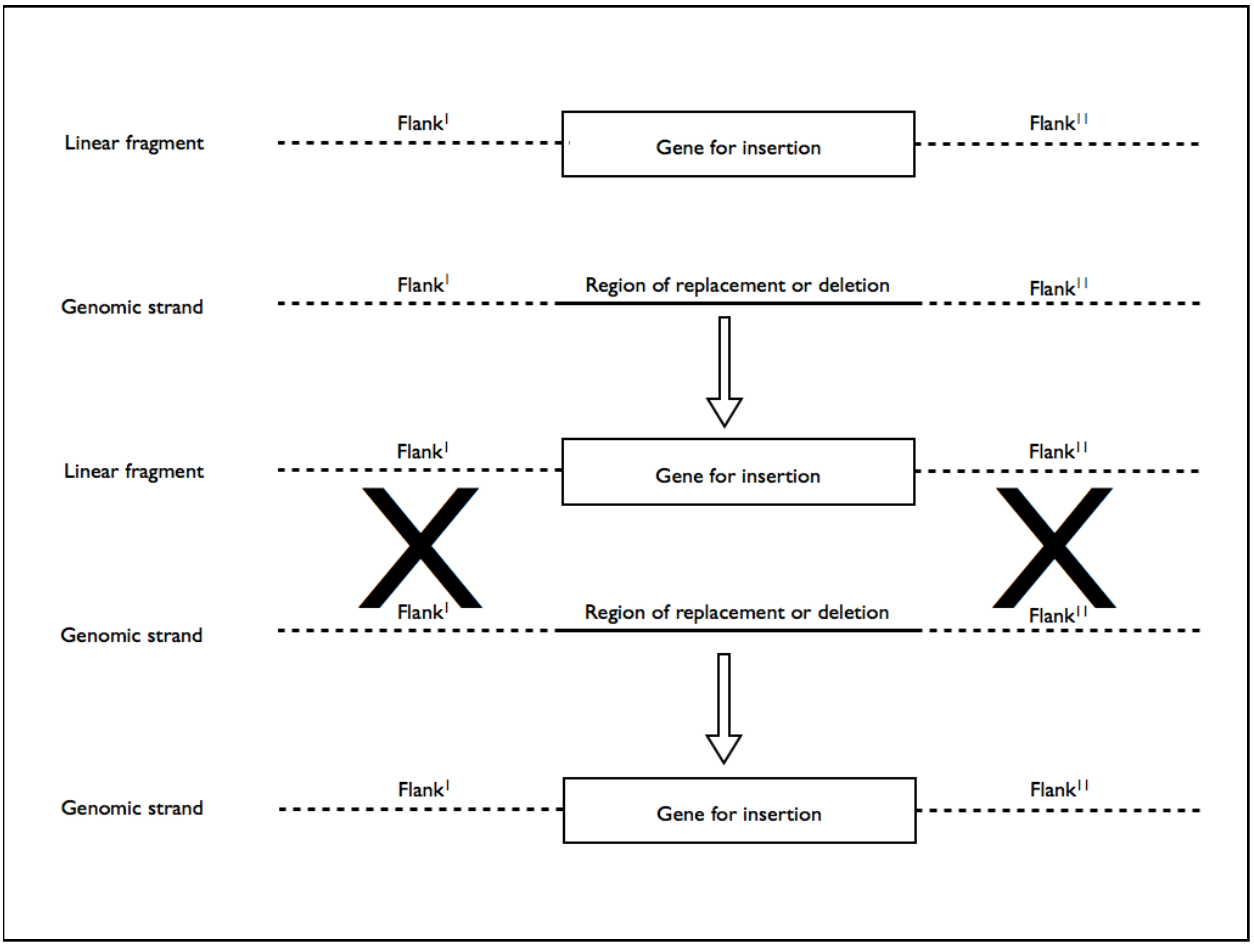

Figure 5: Natural recombination in *Acinetobacter* is dependent on sufficient homology to genomic DNA. Experimentally this means cloning regions flanking the desired area of modification and using them to promote insertion of the carried gene into the chromosome. A clean deletion of the target genomic section is accomplished by fusing flanking pieces together for recombination excluding the targeted section.

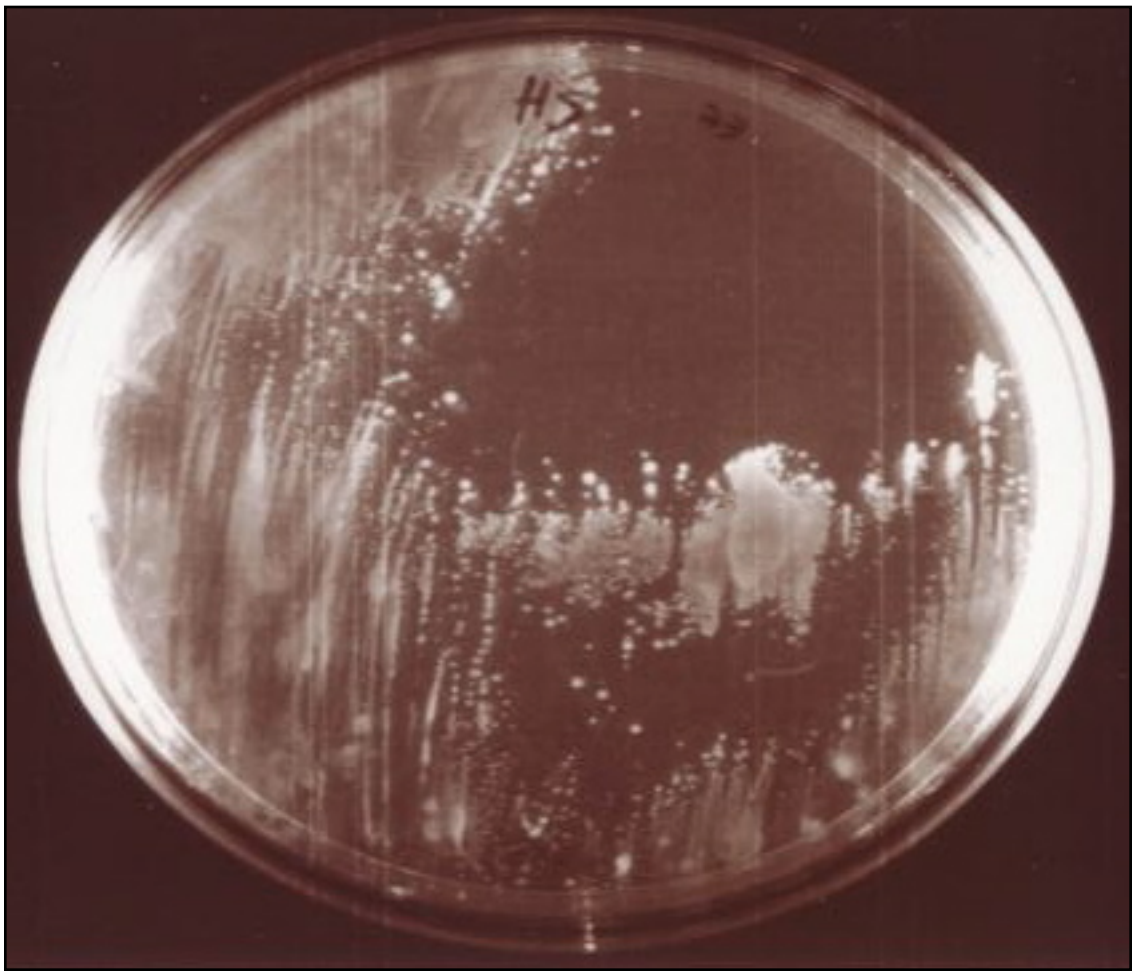

Figure 6: LB heme (10 ug/ul) 6% sucrose (wt/vol) plate with outgrowth from transformation of *A. baylyi hemF* into ADPUGa2 is shown. A large percentage of the growth present does not contain recombinant DNA as evidenced by PCR analysis and lack of a rescued phenotype. Rather, they are believed to be *sacB* loss-of-function mutants.

Chapter 3: A Bacterial CPO Knockout System

Overview

The following outlines the construction of an *Acinetobacter baylyi* ADP1 *hemF* knockout used as an *in vivo* system for the identification of bacterial CPO's, particularly the oxygen-independent HemN enzyme.

Materials and Methods

The plasmids and bacterial strains from construction of the *hemF* knockout are shown in Table 1. Restriction digests were run at 37˚C for one hour unless otherwise specified. All heme media contains 10ug/ml of heme in DMSO.

(Vector 1)

PCR amplification

Construction of the first pUGa vector began by cloning and PCR amplification of 500bp flanking regions immediately upstream and downstream of the ADP1 *hemF* start and stop sites (Figure 7). The upstream flanking region (+500) was cloned with a HindIII restriction site at the +500 base-pair position and a BamH1 restriction site at the +0 base-pair position. The downstream flanking region (-500) was cloned with an EcoR1 restriction site at the -500 base-pair position and a BamH1 restriction site at the -0 base-pair position. PCR amplification of these pieces was performed with *Pfu* Turbo DNA polymerase using the suggested protocol (Stratagene). Primers are shown in table 3. PCR reactions were subjected to 95˚C for 3 minutes and then to 30 cycles; the first three consisted of 45 seconds at 95˚C followed by 1 minute at 55˚C then followed by 1

minute at 72˚C, the last 27 cycles consisted of 30 seconds at 95˚C followed by 30 seconds at 65˚C then followed by 1 minute at 72˚C, all reactions then finished with 10 minutes at 72˚C followed by 4˚C until stopped. Both amplification reactions (+500 and -500) were cleaned up using a standard PCR purification protocol with 50ul used in the elution (Qiagen PCR Purification Kit).

Restriction digest and product ligation

A sequential digest of EcoR1 and HindIII was required for the pUC19 [77] backbone and a sequential digest of HindIII and BamH1 was required for the +500 region. A standard double digest of EcoR1 and BamH1 was used in preparation of the -500 region (New England Biolabs). All digestion reactions were cleaned up with a PCR purification protocol using a 30ul elution (Qiagen). A three piece ligation was run for 30 minutes at 16˚C and then for 1 hour at room temperature. The ligation mix was then transformed into chemically competent *E. coli* GC5 cells (50ul) and grown overnight at 37˚C on LB ampicillin (50ug/ml) plates. Two recombinants were identified by rapid screening [78]. These two recombinant products were grown overnight in Circlegrow media (MP Biomedicals) with ampicillin and the recombinant plasmid was purified using a standard plasmid isolation protocol (Roche High Pure Plasmid Isolation Kit). Plasmid purification of isolated plasmid yielded approximately 6ug to 12ug of plasmid DNA. This plasmid has been designated pUGa1.

(Vector 2)

Introduction of the sacB kanamycin cassette

The *sacB-Km* cassette from the plasmid PRMJ1 [76] was excised by BamH1 (New England Biolabs) digestion and isolated by gel purification on a 1% agarose TBE gel using a standard gel extraction protocol (Qiagen: QIAquick Gel Extraction Kit). The pUGa1 vector was also digested with BamH1 and then cleaned up as previously

described. A 16˚C overnight ligation of the digested pUGa1 vector with the purified *sacB-Km* cassette was transformed into *E. coli* BL21 cells (25ul; Stratagene) and grown overnight at 37˚C on LB kanamycin (30ug/ml) plates. Successful recombination was evidenced by growth on kanamycin and confirmed by removal of the *sacB-Km* cassette via BamH1. This plasmid has been designated pUGa2.

Protocol for plasmid transformation into chemically competent E. coli

Plasmid transformation of plasmid DNA into competent *E. coli* cells for amplification, verification, and purification of plasmids used in this study was performed using a protocol modified from a published transformation protocol (Stratagene). Chemically competent *E. coli* stored at -80˚C were thawed on ice. Fifty microliters (50ul) of cells were aliquoted to prechilled Falcon 2063 polypropylene tubes. Note: 25ul of cells can also be used. If purified plasmid is being transformed, 1ul of plasmid DNA was added to cell aliquot. If a ligation is being transformed, more DNA can be added. Up to 10ul of ligation reaction were used for this study. Cells and added DNA were mixed gently by swirling the tubes and incubated on ice for 30 minutes. Transformation reactions were then heat pulsed at 42˚C for 45 seconds and placed on ice for 2 minutes. 500ul (or 250ul is 25ul of cells were used) of media preheated to 42˚C was then added to reactions and they were incubated at 37˚C for 1 hour. After one hour cells were plated onto appropriate selective plate (maximum of 300ul per plate).

(Transformation into ADP1)

Creation of ADPUGa2

Wild type ADP1 was grown overnight at 37˚C in 5mL of LB media with 10mM succinate. After overnight growth, 10uL of 1M sodium succinate was added (to induce log phase growth) to the culture and incubated for an additional 30 minutes. A single

Sca1 restriction from the pUC19 backbone was used for linearization of the pUGa2 vector. Sca1 digestion was followed by heat inactivation at 80˚C for 20 minutes. The transformation was performed by dropping 1ul of sodium succinate spiked culture onto a LB+heme (10ug/ml) plate (~10 drops per plate). Without allowing the drops of culture to dry, 1ul of linearized pUGa2 vector was dropped onto the plate on top of the drops of culture. These 2ul drops were then allowed to dry and the plates were grown overnight at 37˚C in a dark incubator. Selection for recombinant cells (ADPUGa2) was performed on LB kanamycin (30ug/ml)+heme (10ug/ml) plates. This was accomplished by plating the overnight growth from each 2uL drop onto individual plates and allowing them to grow at 37˚C until colonies appeared (~36-48 hours). Note: It is important to avoid plating too many cells on each selective plate. Too many cells on a plate can result in false-positive growth due to depleted antibiotic. Recombinant insertion of the *sacB*-Km^r cassette was confirmed by PCR analysis of the flanking regions and inserted gene cassette was performed using the +500 sense primer and -500 anti-sense primer (Table 3). The resulting fragment (Figure 8) was digested with BamH1 and Nde1 to yield fragments of the appropriate size (Figure 9).

Creation of ADPUGa1

The formation of a second ADP1 *hemF* deletion strain was performed by the same procedure outlined above using ADPUGa2 as the recipient strain for transformation with Sca1 linearized pUGa1 vector. Transformation was again performed on LB+heme plates. Selection for recombinant cells (ADPUGa1) utilized the *sacB* gene in the ADPUGa2 strain by plating on LB+heme plates with 5% sucrose (wt/vol).

Results

The construction of the ADP1 *hemF* deletion resulted in the creation of two plasmids (pUGa1 and pUGa2) and two unique strains of ADP1 (ADPUGa1 and ADPUGa2). Strain ADPUGa1 contains a clean *hemF* deletion while strain ADPUGa2 possesses the 3.7kb *sacB-Km* cassette in place of the deleted *hemF* gene. Both knockout strains show the *ΔhemF* phenotype as heme auxotrophs. Additionally, strain ADPUGa2 shows resistance to kanamycin and lethal sensitivity to sucrose as expected with the introduction of the *sacB-Km* cassette.

Since the *hemF* knockout results in heme auxotrophy, it is clear the *hemN*-like putative CPO (ACIAD0432) possessed by *A. baylyi* lacks functional CPO activity under these conditions, thus annotation of this gene as a CPO is an error (see Discussion below). The ACIAD0432 gene is a 1.16kb sequence that codes for a 385 amino-acid radical SAM family protein. A BLAST of 0432 identifies genes with significant sequence homology (>74%) in 17 other *Acinetobacter* species (14 of which are classified as CPOs). These proteins are nearly identical in sequence to 0432 which would seem to place these genes into this family of CPO-like radical SAM enzymes with no CPO activity.

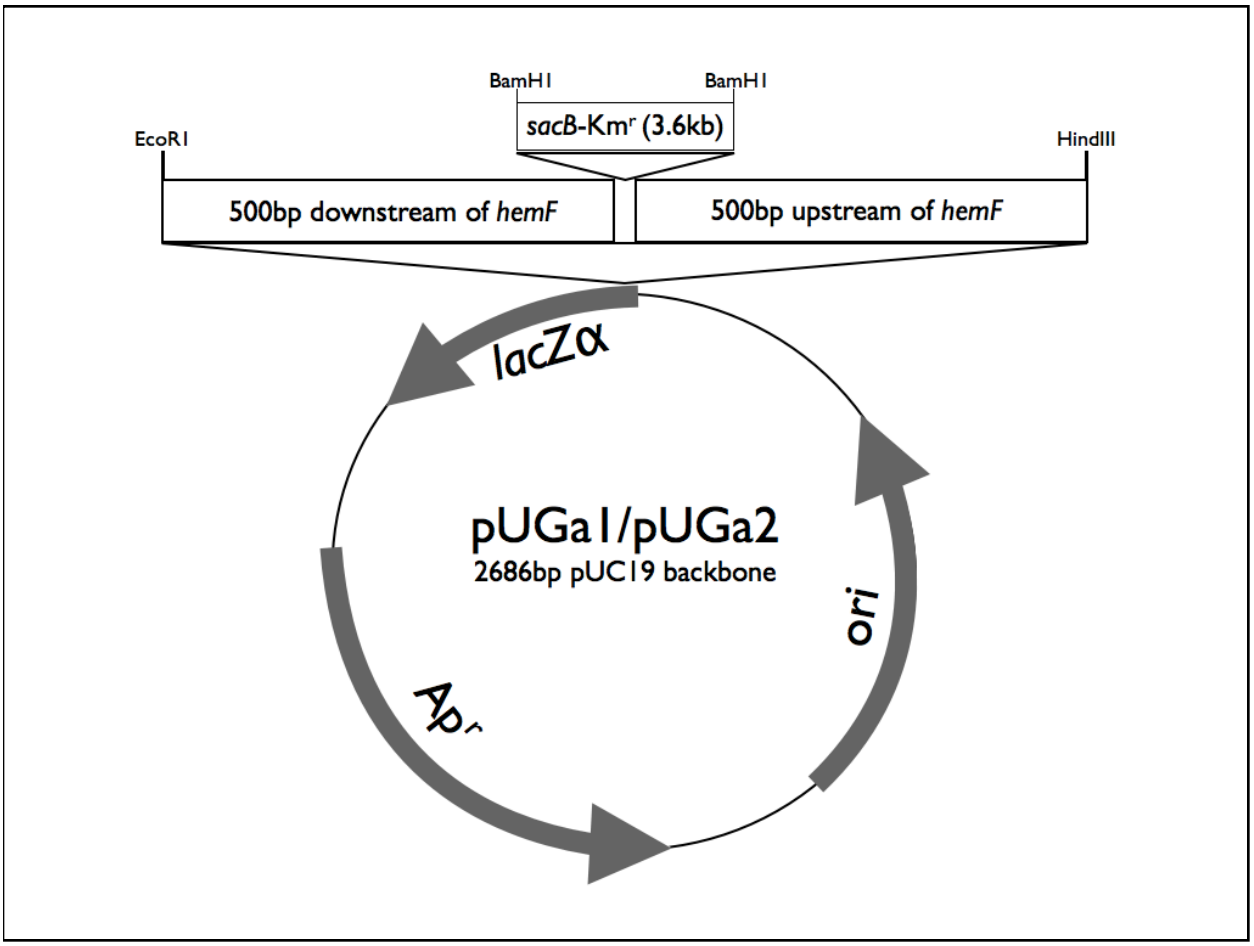

Figure 7: Plasmid map of plasmids used in generation of *hemF* knockout. NOTE: *sacB*-Km^r cassette shown is only in pUGa2, pUGa1 contains only the 500bp flanking regions in the pUC19 backbone.

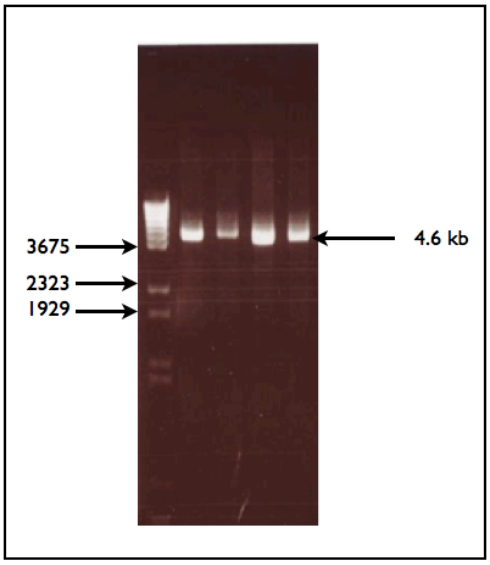

Figure 8: *hemF* substitution with *sacB*-Km^r cassette was confirmed by PCR of genomic DNA from resulting recombinant colonies. PCR product from 4 different recombinant cultures is shown above. Note: Observed difference in the four products is due to quantity of DNA produced in the PCR.

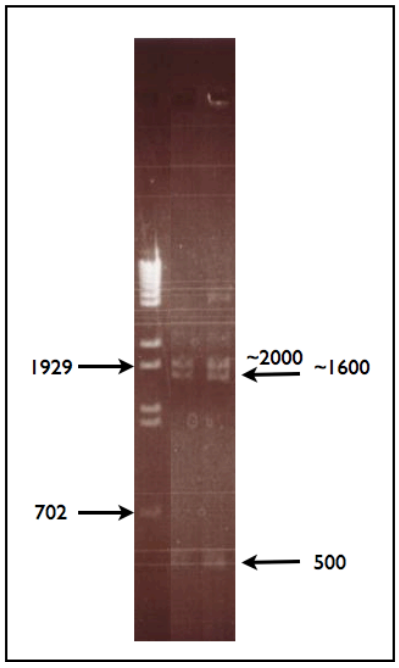

Figure 9: A BamH1 and Nde1 double digest of the *sacB*-Km^r cassette and flanking regions from the PCR shown in figure 7. The digest resulting in the *sacB* gene at ~2000 bp, the Km^r cassette at ~1600 bp, and the two flanking regions at 500 bp.

Table 2: Plasmids and ADP1 strains from construction of knockout.

	Genotype and/or Phenotype	***Source/ Reference***
Plasmids		
pUC19	cloning vector	[66]
PRMJ1	plasmid carrying *sacB-Km* cassette	[67]
pUGa1	pUC19 with *hemF* flanking regions cloned into multiple cloning site	This study
pUGa2	pUGa1 with *sacB-Km* cassette cloned into BamH1 site between +500 and -500 flanking regions	This study
ADP1 Strains		
Wild-Type	Wild-Type	[62]
ADPUGa1	*ΔhemF*	This study
ADPUGa2	*ΔhemF* with 3.6kb *sacB-Km* cassette in place of *hemF* gene	This study

Table 3: Primers used in knockout construction. Respective restriction sites are indicated and shown in brackets.

Primer	***Sequence***
+500 bp	Sense (HindIII): GTT CTT [AAG CTT] TCG CTT CTC TGA AAT GTG A-sense (BamH1): GTT CTT [GGA TCC] AAC ATA AAA CCA TAA AAA AAG TAA G
-500 bp	Sense (BamH1): GTT CTT [GGA TCC] TAT TCG CTT GCA AAT GAG ACT ATT TC A-sense (EcoR1): GTT CTT [GAA TTC] AAA AAG TTT AAT CCT TTT GTG GCA GG

Chapter 4: hemF Knockout Complementation

Overview

The previously described hemF knockout in *Acinetobacter* ADP1 is utilized as a system for identification of CPOs. Recombinant techniques are used to rescue the knockout system with *Acinetobacter hemF.*

Materials and Methods

The ADPUGa2 strain containing the *sacB-Km*^{r} cassette in place of the *hemF* gene was used for all complementation experiments. The vector pUGa1 described above was used to generate a vector for rescue with *E. coli hemN*. New primers used for the following PCR reactions are shown in table 4.

(Complementation with Positive Controls)

PCR reactions

Phenotype rescue with *A. baylyi* ADP1 *hemF* was accomplished by PCR amplification of the *hemF* gene along with the flanking regions described above directly from ADP1 genomic DNA. The entire 1981bp piece was amplified using the +500 sense primer and the -500 anti-sense primer highlighted in table 3. This amplification was performed with Herculase DNA polymerase using the suggested protocol (Stratagene). PCR reactions were run under the same conditions as described above (chapter 3) except for a lengthened extension time of two minutes rather than one. *E. coli hemN* was cloned with BamH1 restriction sites on both ends for insertion into the BamH1 site

on the pUGa1 vector. PCR reactions were run using pTrcHis (Invitrogen) containing the target gene as template DNA. Reactions were again performed with Herculase DNA polymerase according to the suggested protocol (Stratagene). The PCR program for these reactions consisted of 3 minutes at 95˚C followed by 30 cycles. All 30 cycles consisted of 1 minute at 95˚C followed by 1 minute at 52˚C followed by a 1:23 minute extension time at 72˚C. Completion of the 30 cycles was followed by 10 minutes at 72˚C and then 4˚C until stopped.

Restriction digest, product ligation, and plasmid transformation

PCR product containing ADP1 *hemF* and the flanking regions was not modified any further and will be further discussed below. The PCR product containing *E. coli hemN* with terminal BamH1 sites was purified up using a standard PCR purification kit with 50ul elution (Qiagen PCR Purification Kit). A one hour digest at 37˚C was performed with BamH1 (New England Biolabs) on both the *hemN* amplification and the pUGa2 vector. The vector was further treated with antarctic phosphatase (New England Biolabs) for 15 minutes at 37˚C to prevent re-circularization of the vector. All digest reactions were cleaned up with the same PCR purification kit as before. An overnight ligation was performed at 16˚C using a standard T4 DNA ligase protocol (New England Biolabs). The ligation reaction contained equal (8ul) parts pUGa2 vector and *hemN* insert. 5ul of the overnight ligation was transformed into GC10 chemically competent *E. coli* (50ul) and grown overnight on LB ampicillin plates. Colonies present indicated an insertion of the *hemN* gene into the vector but needed further restriction digest to screen for the proper orientation of the inserted gene. This was done by a one hour digestion at 37˚C with Sca1 (New England Biolabs). Sca1 restriction sites were identified on both

the vector backbone and within the *hemN* and allowed for an assessment of gene orientation within the vector (Figure 10).

Transformation into ADPUGa2

Introduction of A. baylyi ADP1 *hemF*

The *hemF* knockout was rescued with the native *hemF* previously removed from the chromosome. The PCR product containing the *hemF* gene and flanking regions was directly transformed without any modification (though transformation with PCR product cleaned up with a PCR clean-up kit yielded similar results). A 5ml overnight culture of ADPUGa2 was grown in LB with kanamycin (30ug/ml) and heme (10ug/ml). This culture was spiked with 10ul of 1M succinate for 30 minutes prior to plating. The transformation was performed on LB heme (10ug/ml) plates 1ul drops of culture (~15 per plate) spread out over the plate. One microliter (1 ul) drops of PCR product were then dropped on top of the drops of culture and the drops were allowed to dry together and incubated in the dark overnight at 37˚C. Selection against the *sacB* gene in the ADPUGa1 strain (which should be crossed out with recombination) was performed on LB heme plates with 5% succrose (wt/vol) as described above. The selection plates were grown overnight and resulting colonies were plated on LB (no heme) to indicate a rescue in the heme biosynthesis knockout phenotype. Confirmation of correct recombination was performed by PCR amplification of the gene and flanking regions from genomic DNA of resulting *A. baylyi* strain (a regeneration of wild-type ADP1) (Figure 11).

Results

As previously stated, the phenotype was successfully rescued with the native *hemF* gene from ADP1. This was accomplished in spite of the poor selection efficiency of the sucrose/*sacB* selection method, though it was clear these issues affected the experiment. This strain, which is a regeneration of wild-type ADP1 displays a rescued phenotype and is capable of growing in the absence of exogenous heme. Additionally, it shows a loss of kanamycin resistance as seen in the ADPUGa2 knockout strain. Phenotype rescue with *E. coli hemN* was unsuccessful under the current methods.

Discussion and Future Direction

The growing volume of available genomic data has afforded researchers an invaluable resource and given rise to many genes being classified and annotated based on sequence homology with already characterized proteins. However these annotations are often based on little or no supporting biochemical data and many times this leads to correct identification of novel genes and appropriate designation of the proteins they encode. But as has been shown previously and by this study, relying on homology based annotation alone has lead to broad mis-annotation of bacterial oxygen-independent CPOs. The CPO knockout highlighted in this study should help advance our ability to not only identify proper CPO enzymes but also to classify families of CPO-like enzymes from the radical SAM family that do not have CPO activity as well as identify novel CPO enzymes.

It was shown that a *hemF* deletion in *A. baylyi* ADP1 is lethal unless exogenous heme is present and that this phenotype can be rescued by reintroduction of CPO into the genome. Additionally, the plasmid DNA necessary to carry this out in the naturally

competent deletion has been cloned and characterized. The lethality of the gene deletion under these conditions classifies ACIAD0432 as encoding for a CPO-like radical-SAM protein with no CPO activity. Using this characterization, homology data and gene cluster data from other genomes can be used to help characterize other related radical-SAM proteins. ACIAD0432 belongs to the heat shock *dnaK* gene cluster and is classified as a CPO-like radical SAM enzyme clustered with the nucleoside-triphosphatase RdgB (SEED). The *dnaK* gene cluster is associated with hyperosmotic and heat shock response through prevention of aggregation of stress-denatured proteins as well as de-aggregation of stress-denatured proteins [79]. However, genes within the cluster have not been characterized. As previously stated 17 different *Acinetobacter* genomes have nearly identical proteins to ACIAD0432 which can also be assumed to lack CPO activity under these conditions and fall into the same CPO-like radical SAM family. The data on 0432 can also help shed light on other genes clustered with RdgB which belong to the CPO-like radical SAM family 1. Notably, *P. aeruginosa* contains a 1.16kb gene (PA0386) classified as a *hemN*. This protein has 53% homology to 0432 and is found within the heat shock *dnaK* gene cluster. Within this gene cluster family, similar CPO-like radical-SAM proteins are found in *R. sphaeroides*, *E. coli*, *Buchnera aphidicola*, and *H. influenzae* (Table 5). None of these genes have been characterized and further research will be necessary to determine their function in heat shock response.

Moving forward, it will be necessary to integrate a new selective measure into the knockout system. Negative selection against the *sacB* gene proved highly inefficient and made identification of recombinant colonies nearly impossible. It is suggested that

an alternative antibiotic resistance be incorporated into the pUGa1 vector to replace the kanamycin cassette crossed-out during recombination. This would allow for better selection of recombinant colonies and also help prevent contamination of the experiment. Incorporation of this resistance could be in the *sacB*-Km^r model and 'piggy-back' the resistance cassette in with the gene of interest (Figure 12). This should allow for a superior selection of recombinant colonies during outgrowth and maximize the effectiveness of this system for the identification of CPO enzymes.

Table 4: Primers used in knockout complementation. Respective restriction sites are indicated and shown in brackets. Note: complementation with ADP1 *hemF* was done using primers described in Chapter 2 (Table 3).

Primer	***Sequence***
EC HN	Sense (BamH1): GTT CTT [GGA TCC] ATG TCT GTA CAG CAA ATC GAC A-sense (BamH1): GTT CTT [GGA TCC] TTA AAT CAC CCG AGA GAA CTG C

Table 5: Genes associated with the CPO-like radical-SAM enzyme family clustered with the nucleoside-triphosphatase RdgB. Percent identity to ACIAD0432 (YP_045195.1) from BLAST sequence comparison is given.

Gene	***Organism***	***Previous annotation***	***%***	***Accession number***
PA0386	*P. aeruginosa*	*hemN*	53	NP_249077.1
RSP_1224	*R. sphaeroides*	*hemN*	35	YP_354306.1
yggW	*E. coli*	predicted oxioreductase	49	NP_417430.1
yggW	*B. aphidicola*	hypothetical protein	38	NP_240356.1
HI0463	*H. influenzae*	*hemN*	49	NP_438624.1

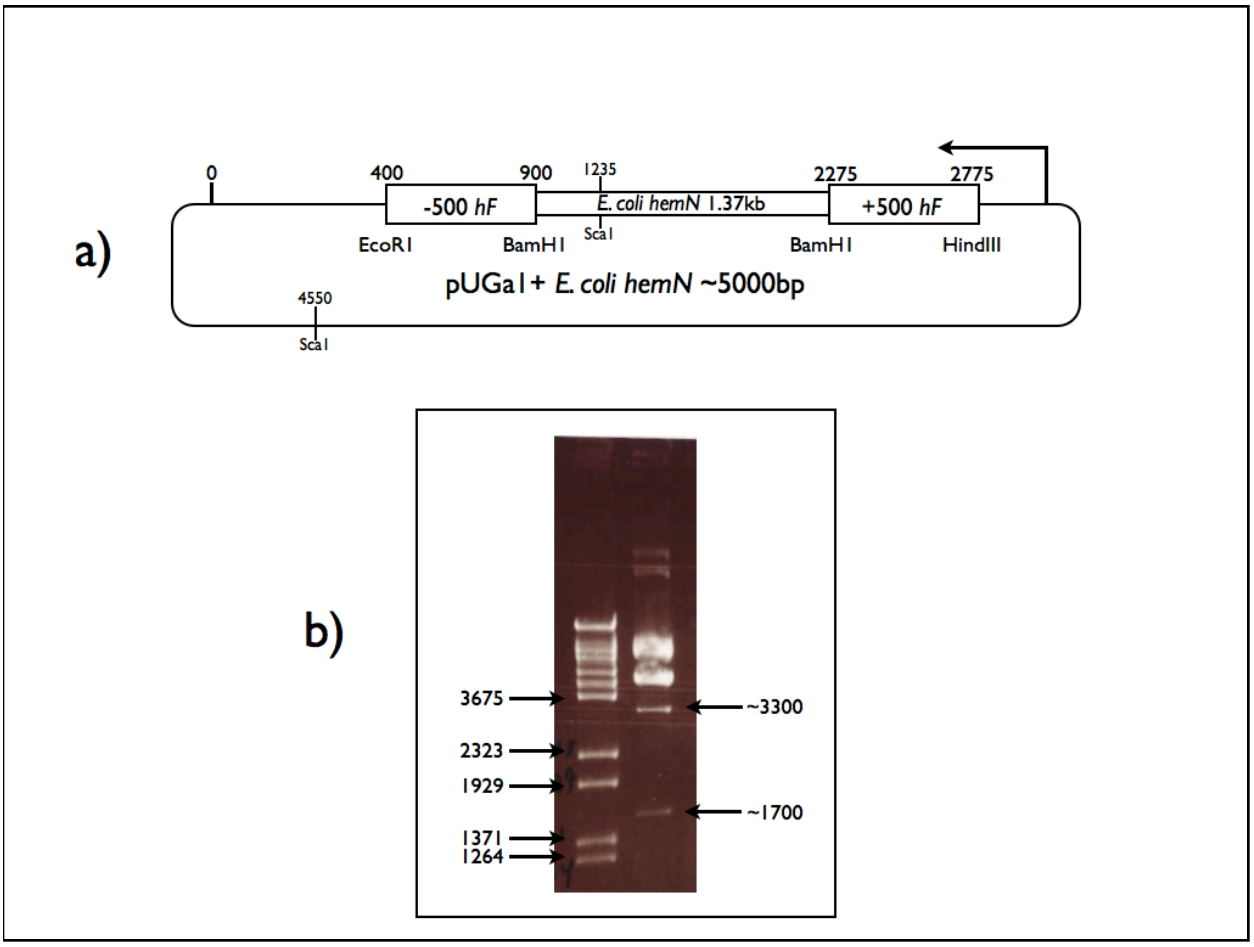

Figure 10: Cloning strategy for insertion of *E. coli hemN* into DNA vector for recombination into *A. baylyi* genome. a) Plasmid map showing *hemN* gene within flanking regions and relevant restriction sites. b) Ethidium bromide staining of 1% agarose gel in TBE indicates proper orientation of *hemN* gene in carrier vector; bands at ~3.3kb and ~1.7kb are indicative of an in frame insertion of the gene.

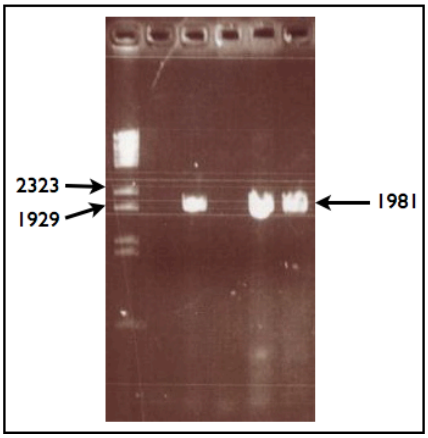

Figure 11: PCR analysis of *hemF* gene with flanking regions from *A. baylyi* strain with phenotype rescued by re-introduction of *hemF* gene. Confirmation of successful transformation is based on presence of appropriate PCR product (~1981 bp; 981 bp gene and 1000 bp flanking region) and absence of PCR product at 4.6 kb indicating presence of *sacB*-Km^r cassette from *hemF* substitution strain ADPUGa2 (Figure 7).

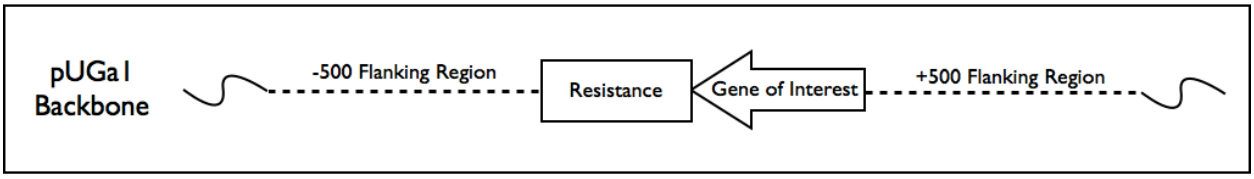

Figure 12: Proposed model for incorporation of new antibiotic resistance into the pUGa1 vector to be used for recombinant complementation experiments with the ADPUGa2 knockout strain.

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