ISOLATION OF A Pseudomonas aeruginosa ASPARTATE TRANSCARBAMOYLASE MUTANT AND THE INVESTIGATION OF ITS GROWTH CHARACTERISTICS, PYRIMIDINE BIOSYNTHETIC ENZYME ACTIVITIES, AND VIRULENCE FACTOR PRODUCTION

Heidi Carol Hammerstein, B.S.

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APPROVED:

Gerard A. O’Donovan, Major Professor
Robert C. Benjamin, Minor Professor
Kathleen L. Bishop, Committee Member
Arthur J. Goven, Biology Department Chair
Sandra L. Terrell, Dean of the Robert B. Toulouse School of Graduate Studies
The pyrimidine biosynthetic pathway is an essential pathway for most organisms. Previous research on the pyrimidine pathway in *Pseudomonas aeruginosa* (PAO1) has shown that a block in the third step of the pathway resulted in both a requirement for exogenous pyrimidines and decreased ability to produce virulence factors. In this work an organism with a mutation in the second step of the pathway, aspartate transcarbamoylase (ATCase), was created. Assays for pyrimidine intermediates, and virulence factors were performed. Results showed that the production of pigments, haemolysin, and rhamnolipids were significantly decreased from PAO1. Elastase and casein protease production were also moderately decreased. In the *Caenorhabditis elegans* infection model the nematodes fed the ATCase mutant had increased mortality, as compared to nematodes fed wild type bacteria. These findings lend support to the hypothesis that changes in the pyrimidine biosynthetic pathway contribute to the organism’s ability to effect pathogenicity.
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Pseudomonas aeruginosa is a prokaryotic aerobe found commonly in water and soil. It is a Gram-negative, rod, having a fully sequenced genome of 6,264,403 base pairs (Stover et al. 2000). Under normal conditions P. aeruginosa is harmless to humans. However, it is an opportunistic pathogen, and can cause deadly infections in burn victims and cystic fibrosis patients. It has also recently been found to be living very happily under anaerobic conditions inside the human lung (Hassett et al. 2002). It is the goal of this research to identify a potential target in P. aeruginosa, which would kill the bacterium inside its host, or at least render it harmless.

Pyrimidine synthesis is a basic function essential for DNA and RNA synthesis, and necessary for the survival of most organisms (O’Donovan and Neuhard 1970; Patterson and Carnright 1977; Doremus 1986; Denis-Duphil 1989). P. aeruginosa utilizes the pyrimidine biosynthetic pathway shown in Figure 1. The genes involved and the enzymes they encode are shown in the pathway illustration. Glutamine (Gln) or the ammonium ion, adenosine triphosphate (ATP) and bicarbonate are utilized by carbamoylphosphate synthase (CPSase) to form carbamoylphosphate, glutamate (Glu) or water, and adenosine diphosphate (ADP). Carbamoylphosphate and aspartate (Asp) are catalyzed by aspartate transcarbamoylase (ATCase) to form N-carbamoylaspartate. Dihydroorotase (DHOase) catalyzes the dehydration, which circularizes N-carbamoylaspartate to form dihydroorotate (DHO) (Fig. 1a). Dihydroorotate dehydrogenase (DHODhase) oxidizes DHO (NAD+/NADH-dependent) to form orotate (OA). OA and phosphoribosyl pyrophosphate (PRPP) are utilized by orotate phosphoribosyl transferase.
Figure 1a  Pyrimidine Biosynthetic Pathway – Bicarbonate Through Dihydroorotate
Dihydroorotate dehydrogenase (DHOdehase) pyrD

PRPP PPi

Orotate phosphoribosyl transferase (OPTase) pyrE

Uridylate (UMP) pyrF

Figure 1b Pyrimidine Biosynthetic Pathway – Dihydroorotate Through Uridylate
(OPRTase) to form orotidylate (OMP). Orotidylate decarboxylase catalyzes the decarboxylation of OMP to uridylate (UMP) (Fig. 1b). The phosphorylation of UMP by ATP is catalyzed by UMP kinase (using Mg$^{2+}$ cofactor) to form uridine diphosphate (UDP). Nucleoside diphosphate kinase (ndk) catalyzes the phosphorylation of UDP by ATP to form uridine triphosphate (UTP). UTP can then be incorporated into RNA molecules, or used to synthesize cytidine triphosphate (CTP). CTP synthetase catalyzes the addition of an amine group to UTP to form CTP (Fig. 1c). Both UTP and CTP are used in the cell for RNA synthesis or converted to dNTPs and ultimately used for DNA synthesis (Kornberg and Baker 1992).

The pyrimidine biosynthesis pathway is regulated in two ways. 1) Transcriptional control: The accumulation of the end products, UTP and CTP, decreases intermediate enzyme activity (Isaac and Holloway 1968; O’Donovan et al. 1989). There is a trickle of enzyme production, but transcription is down regulated at the DNA level. 2) Enzymatic control: Our laboratory hypothesizes that OA induces pyrE and pyrF (Kumar 2002; Patel 2001), which prevent feedback inhibition. A representation of the approximate locations of these and other pyrimidine pathway regulatory genes in wild-type P. aeruginosa (PAO1) (Holloway et al. 1979) is shown in Figure 2. The data corresponding to each gene are included in Table 1 (Stover et al. 2000).

Our laboratory has characterized many of the genes involved in the pyrimidine biosynthetic pathway of pseudomonads. Pyrimidine mutant strains of PAO1 have been produced for the study of their capacity for pyrimidine synthesis and pathogenicity. These strains have blocks at various steps of the pyrimidine biosynthetic pathway, thus they are auxotrophic organisms, unable to produce their own RNA. However, they are able to survive and continue to function through the addition of exogenous uracil, which is utilized by the organism in the
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<td>Adaptation, protection; Nucleotide BAM</td>
</tr>
<tr>
<td>crc</td>
<td></td>
<td>6002120</td>
<td>6003420</td>
<td>PA5332</td>
<td>catabolite repression control protein</td>
<td>Carbon compound catabolism; Energy metabolism</td>
</tr>
<tr>
<td>pyrC2</td>
<td></td>
<td>6233117</td>
<td>6243417</td>
<td>PA5541</td>
<td>probable dihydroorotase</td>
<td>Nucleotide BAM</td>
</tr>
</tbody>
</table>

Base pair locations are given from 0 minutes point in Figure 2. BAM = biosynthesis and metabolism.

Table 1. Pyrimidine Biosynthesis and Metabolism Genes in the *P. aeruginosa* Genome
salvage pathway (Beck 1995). It is the presence of exogenous uracil that activates the salvage pathway, even in organisms without a block in the biosynthetic pathway. The salvage pathway is illustrated in Figure 3. Initially, the exogenous uracil (U) has to get through the cell membrane. *P. aeruginosa* facilitates this transport with uracil permease (*uraA*), or cytosine permease (*codB*) in the case of exogenous cytosine (C), or C4-dicarboxylate transport protein (*dctA*) in the case of exogenous orotate (O). These are transmembrane proteins that allow the small molecules to transverse *P. aeruginosa*’s cell membranes. U is converted to UMP with *upp*, which encodes uridylate phosphoribosyl transferase (Fig. 3). With these two steps the block in the pyrimidine biosynthetic pathway is averted and the biosynthesis of RNA can continue as normal (Fig. 1c). C is converted to U with *codA* which encodes cytosine deaminase. Once inside the cell O can enter the pyrimidine biosynthetic pathway at *pyrE* (Fig. 1b).

The amino acid arginine is produced via a pathway that is an offshoot of the pyrimidine biosynthetic pathway. Arginine synthesis, as illustrated in Figure 4, begins with carbamoylphosphate and the addition of ornithine and ornithine carbamoyltransferase (*argF*) to form citrulline. Citrulline, aspartate and argininosuccinate synthase (*argG*) react to form argininosuccinate. Argininosuccinate and argininosuccinate lyase (*argH*) react to form arginine.

**Hypothesis**

Among the pyrimidine mutant strains characterized in our laboratory is a double knockout (DKO) of DHOase (*pyrC*, *pyrC2*) strain PAO1DB92 (Brichta et al. 2004). Results indicate that the *pyrC* and *pyrC2* are influential in *P. aeruginosa*’s ability to produce virulence factors (Brichta 2003). This may be related to the measured imbalance in pyrimidine biosynthetic intermediates in the DHOase mutant. Without functional *pyrC* and *pyrC2* there is a build-up of carbamoylaspartate (CAA), because there is no DHOase being produced to convert
Pyrimidine Synthesis by Salvage

Figure 3. The Salvage Pathway and the Interactions with the Biosynthetic Pathway
Figure 4. The Arginine Pathway and the Interactions with the Pyrimidine Biosynthetic Pathway
the CAA to DHO (Fig. 1a). It is hypothesized that this build-up of CAA is the cause of an observed decrease in pathogenicity of the \textit{pyrC} DKO strain. In order to determine if it is CAA that is responsible for decreased pathogenicity, it was necessary to create a \textit{pyrBC\textasciiacute} knockout. \textit{pyrBC\textasciiacute} encodes the second enzyme in the pathway aspartate transcarbamoylase (ATCase), necessary for the conversion of carbamoylphosphate and aspartate into CAA (Fig. 1a). The question is the following: Does the build-up of CAA in PAO1DB92 cause a decrease in the ability to export virulence factors as compared to the wild type? The null hypothesis ($H_0$) is that the CAA build-up is the cause for a decreased ability of PAO1DB92 to export virulence factors, therefore, a \textit{pyrBC\textasciiacute} mutant would have the same virulence as PAO1. The alternate hypothesis ($H_A$) is that the CAA build-up is not the cause for a decreased ability of PAO1DB92 to export virulence factors, therefore, a \textit{pyrBC\textasciiacute} mutant will be less virulent than PAO1.

**Project Overview**

The enzyme to be eliminated from PAO1 is ATCase. ATCase was characterized first in \textit{Escherichia coli} by Gerhart and Holoubek (1967). ATCase is a holoenzyme made up of four trimers, with a total size (for PAO1) of 486-kDa (Bergh and Evans 1993; Schurr et al. 1995). Two 36-kDa \textit{pyrB}-encoded polypeptide trimers each attached to a trimer of 45-kDa (the product of the attached downstream gene) \textit{pyrC\textasciiacute}-encoded polypeptides. Two separate trimers then form dimers at the center of the ATCase enzyme (Fig. 5). \textit{pyrC\textasciiacute} encodes a DHOase-like polypeptide, which is inactive in this enzyme. Although the \textit{pyrB} polypeptides are the active portion of ATCase, the enzyme is rendered inactive without the \textit{pyrC\textasciiacute} polypeptides.

In this study a \textit{pyrBC\textasciiacute} mutant strain of PAO1 was created, and characterized to determine the effect of reduced CAA production on the cell physiology. An outline of the project is illustrated in Fig. 6. Project results are compared with those for PAO1DB92 to determine the
Class A ATCase: ~480 kDa

Class B ATCase: ~300 kDa

Class C ATCase: ~100 kDa

Fig. 5. The three classes of bacterial ATCases. Used with permission form Brichta, 2003.
role of pyrimidine intermediates with respect to the pathogenicity of *P. aeruginosa*.

### Cloning Strategy

The *pyrBC'* mutation was accomplished using the methods of DNA deletion, and insertional inactivation using a gentamicin cassette (Schweizer 1993). The *pyrBC'* sequence is made up of *pyrB* and *pyrC'*, which overlap each other by 4 base pairs (bp) (Stover et al. 2000). Together *pyrBC'* are a total of 2272 bp (Fig. 7). The plasmid pA10, created in our laboratory (Vickrey 1993), was used as a starting point for the mutation of *pyrBC'*.

pA10, illustrated in Figure 8, was composed of a *Xho*I fragment cut from PAO1 genomic DNA and the cloning vector pUC19. The *Xho*I fragment, containing *pyrBC'*, was inserted at the *Sac*I site of the pUC19 polylinker. pA10 was digested with *Sac*I and the large fragment re-circularized to produce pHH01 (Fig. 8). This resulted in a deletion of 1191 bp, severely truncating the *pyrC'* sequence, leaving approximately 500 bp of the 5' end and 500 bp of downstream genomic PAO1, either section available for recombination on the 3' end of the gene. Figure 7 shows the omitted *pyrC'* sequence highlighted in orange. pHH01 was then digested with *Nco*I and *Eco*RV, resulting in a 417 bp excision from the 3' end of *pyrB*, leaving approximately 500 bp homologous to the wild type available for cross-over on the 5' end of the gene. Figure 7 shows the omitted *pyrB* sequence highlighted in blue. Both deletions totaled 2328 bp. Next, a gentamicin cassette was isolated from the plasmid pGMΩ1, developed by Schweizer (1993). The large fragment of the *Nco*I and *Eco*RV digested pHH01 was ligated with the isolated gentamicin cassette, forming plasmid pHH02 (Fig. 9). The new construct was transformed into *E. coli* DH5α, grown to mid log phase, and screened for the plasmid on gentamicin plates. The plasmid was isolated from the *E. coli* cells and digested with restriction enzymes to verify the correct plasmid size, and placement of the gentamicin cassette. The *pyrB/GmΩ* construct was
Check *E. coli* strains TB2 and DH5α. Check plasmids pA10, pUCP19 and pUCP20. Transform cells and maintain stocks.

Check *P. aeruginosa* strains PAO1 and PAO1DB92. Maintain stocks.

Cut with *Sma* I to isolate 1.7 kb Gm cassette.

Ligate with Gm cassette to form pHH02, cut mutated gene out of pHH02 with *Sac* I and *Sph* I, ligate with linearized pRTP1 to form pHH08.

*Sac* II digest of pA10. Recircularize large fragment to form pHH01. Ncol and *Eco* RV digest of pHH01. Purify large band for ligation with Gm cassette.

Check pRTP1 with restriction enzymes and gel to verify sizes. *Eco* RI digest, blunt and CIP.

Grow pHH08 in DH5α to check plasmid. Transform recombinant plasmid into SM10.

Mate SM10 + pHH08 with PAO1. Perform cross-over selections.

Verify *pyrB* mutant is auxotrophic and has no ATCase.

Assay PAO1 and mutant for pyrimidine pathway products and virulence factors.

Growth Studies for PAO1 vs. *pyrB* mutant.

Southern Blot and hybridization.

Figure 6. Project Overview
Figure 7. DNA and Protein Sequences for pyrB and pyrC'. The sequence shown is from the minus strand of P. aeruginosa in the reading direction (Stover et al., 2000).
Figure 8. Construction of pA10 and pHH01
Figure 9. Cloning Strategy Used to Construct pH02 and pH08
isolated from pH02 with a *Sac*I and *Sph*I digest and ligated into the multiple cloning site (MCS) of pRTP1 (Stibitz et al. 1986), forming pH08 (Fig. 9). pRTP1 was used because of its propensity to undergo homologous exchange. Additionally, pRTP1 does not contain a broad-host-range origin of replication (*oriT*), and cannot be sustained inside *P. aeruginosa* after crossing out of the chromosome. pH08 was transformed into *E. coli* DH5α, which was grown to mid log phase and screened for the plasmid. pH08 was isolated from the DH5α cells and digested with restriction enzymes to verify the presence of the disrupted *pyrB*. pH08 was then transformed into *E. coli* SM10 and mated with PAO1 via biparental mating (Hoang et al. 1998). The SM10 strain contains the Tra genes necessary for the production of mating pili compatible with *P. aeruginosa*. Once inside the PAO1 cells pH08 was able to exchange alleles with the PAO1 genomic DNA, incorporating the disrupted *pyrBC′*, resulting in a *pyrBC′*− strain of PAO1 (HD3β). Figure 10 illustrates the recombination locations between pH08 and PAO1.

**Screening of PAO1 Mutations**

Intermediate screening was performed during PAO1 and pH08 recombination to help increase the amount of desired product and decrease the amount of overall screening required. First, single crossover (SCO) events were selected for, which would include the entire pH08 plasmid within the *P. aeruginosa* genome. These isolated SCO cells were allowed to grow further (in the absence of selection antibiotics) to encourage double crossover (DCO) events. Then the DCO cells, with the desired disrupted gene incorporated and the vector crossed out, were screened for pyrimidine auxotrophy.

It is the configuration of the vector that allows for this selection process. Once recombined, the vector confers its resistance or sensitivity to the organism. Once removed from the genome by recombination, the vector used cannot persist in the organism and thus cannot
Figure 10. Single and Double Crossover Events
affect its phenotype. The relevant phenotypes for the pRTP1 vector and mutated pyrBC' system are gentamicin resistance/carbenicillin resistance (Gm'/Cb') for the SCO, and Gm'/carbenicillin sensitivity (Cb') for the DCO (Fig. 10).

Characterization

Our laboratory has characterized *P. aeruginosa* and its capability to synthesize pyrimidines through the *de novo* and salvage pathways (Vickrey 1993; Linscott 1996; Kumar 2002; Brichta 2003). HD3β was characterized in the same way for its pyrimidine biosynthetic enzymatic activities, pathogen synthesis, and motility capabilities. The following enzymes were assayed for their specific activity: aspartate transcarbamoylase (ATCase), dihydroorotase (DHOase), dihydroorotate dehydrogenase (DHOdehase), orotate phosphoribosyltransferase (OPRTase), orotidylate decarboxylase (OMPdecase), and ornithine transcarbamoylase (OTCase). Characterization of pathogen synthesis includes virulence factors: pyoverdin, pyocyanin, elastase, casein protease, haemolysin, iron chelation and rhamnolipid production. The following assays were performed to observe motility: cross-streak inhibition, twitching, swarming, and swimming.
CHAPTER II
MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used are listed in Table 2 below.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype, Relevant Phenotype, or Function</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>wild type</td>
<td>Holloway et al. 1979</td>
</tr>
<tr>
<td>PAO1DB92</td>
<td>pyrC(^{-}), pyrC2(^{-})</td>
<td>Brichta et al. 2004</td>
</tr>
<tr>
<td>HD3β</td>
<td>pyrB(^{-})</td>
<td>This work</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>lacZ(^{a-})</td>
<td>GIBCO-BRL</td>
</tr>
<tr>
<td>TB2</td>
<td>pyrB(^{-})</td>
<td>Donson et al. 1991</td>
</tr>
<tr>
<td>SM10</td>
<td>helper plasmid for biparental mating</td>
<td>Simon et al. 1983</td>
</tr>
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</table>

<table>
<thead>
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<th>Plasmids</th>
<th></th>
<th></th>
</tr>
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<td>Characterization of <em>E. coli</em> strains</td>
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</tr>
<tr>
<td>pA10</td>
<td>Isolated *P. aeruginosa pyrBC'</td>
<td>Vickery 1993</td>
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<tr>
<td>pGM01</td>
<td>Insertional inactivation of pyrBC'</td>
<td>Schweizer 1993</td>
</tr>
<tr>
<td>pH01</td>
<td>pA10 with deletions from pyrBC'</td>
<td>This work</td>
</tr>
<tr>
<td>pH02</td>
<td>pH01 with Gm cassette in place of .4kb pyrB</td>
<td>This work</td>
</tr>
<tr>
<td>pH08</td>
<td>Mutated pyrBC' cloned into pRTP1</td>
<td>This work</td>
</tr>
<tr>
<td>pRTP1</td>
<td>Enables homologous exchange between plasmid and chromosome</td>
<td>Stibitz et al. 1986</td>
</tr>
</tbody>
</table>

Table 2. Strains and Plasmids Used in This Study

All media recipes below are given for one liter total volume of broth, or in molarities (M) of final concentration in solution. Unless otherwise stated, deionized, distilled, purified water (ddH\(_2\)O) was used for the liter volume. Where plates were needed instead of broth, 16 g of agar was used per liter volume. Where agar was already present in a powdered mix, agar was added to equal 1.6% (w/v) total. Difco® (Detroit, Mich., USA) granulated agar was used when preparing rich medium plates. Difco® Bacto® agar was used when preparing minimal medium plates. Unless otherwise stated, sterilization of all media and components below was accomplished using an autoclave with settings of 121°C and 17 psig for 20 minutes.
Antibiotic components, nutritional components and selection components (expressed in final concentration) used with *E. coli* strains were 100 µg/ml ampicillin (Amp<sub>100</sub>), 25 µg/ml kanamycin (Km<sub>25</sub>), 0.2% glucose (w/v), 1 mM MgSO<sub>4</sub>, 0.001685% thiamine (w/v) or 1.685 µg/ml, 0.1 mM histidine, 5.0 mM adenine pH with 0.1 N HCl (filter sterilized), 0.4 mM isopropylthio-β-D-galactoside (IPTG) (filter sterilized), 0.004% 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal) (2% stock in dimethyl formamide), 0.1265% arginine (w/v) or 126.5 µg/ml. Those used with *P. aeruginosa* strains were 100 µg/ml gentamicin (Gm<sub>100</sub>), 600 µg/ml carbenicillin (Cb<sub>600</sub>), 5, 20, and 40 µg/ml uracil (U<sub>5</sub>, U<sub>20</sub>, U<sub>40</sub>), 40 µg/ml orotate (O<sub>40</sub>), 0.2% glucose (w/v).

The following buffers were made in the method of Sambrook et al. (1989). One half M ethylenediamine tetra acetic acid (EDTA) (pH to 8.0) with NaOH, 1 M tris-HCl (pH 8.0), tris-acetate (TAE): 0.04 M tris-acetate and 1 mM EDTA, TE buffer: 10 mM tris-HCl and 1 mM EDTA, phosphate-buffered saline (PBS): 8 g NaCl, 2 g KCl, 1.44 g Na<sub>2</sub>HPO<sub>4</sub> and 0.24 g KH<sub>2</sub>PO<sub>4</sub>, potassium phosphate buffer (KPB): using K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> mixtures to achieve various pH levels, 20X SSC: 175.3 g NaCl and 88.2 g NaCitrate, 6X Gel-loading buffer I: (modified to contain less indicator dye) 0.1% (w/v) bromophenol blue, 0.1% w/v xylene cyanol FF and 40% (w/v) sucrose.

*E. coli* strains were cultivated in Difco® Luria-Bertani (LB) medium. The LB medium contained 10 g Bacto® tryptone, Bacto® 5 g yeast extract, and 10 g NaCl. Occasionally *P. aeruginosa* strains were also cultivated in Difco® Lennox LB medium with a 10/5/5 recipe, consisting of 10 g tryptone, 5 g yeast extract, and 5 g NaCl. The 10/5/5 ratio was used to minimize deleterious effects of the Na on the subsequent pyrimidine biosynthetic enzyme activity assays.
E. coli strains were also cultivated in E. coli minimal medium (Ecmm) (Gause, 1934; Jost et al. 1972), consisting of 60.3 mM K$_2$HPO$_4$, 33 mM KH$_2$PO$_4$, 7.6 mM (NH$_4$)$_2$SO$_4$, 1.7 mM NaCitrate. When plates were needed, the medium was autoclaved in two portions: half of the ddH$_2$O with the agar, and half of the ddH$_2$O with the salts. The two portions were combined aseptically after sterilization by pouring the salts into the agar very slowly down the wall of the flask to avoid bubbles in the medium. After cooling, nutrients (glucose, MgSO$_4$ and thiamine mandatory) and the appropriate antibiotics and selection components (in the above concentrations) were added.

PAO1 strains were cultivated in Pseudomonas minimal medium (Psmm) (Ornston and Stanier 1966), consisting of 12.5 mM Na$_2$HPO$_4$, 12.5 mM KH$_2$PO$_4$, 0.1% (NH$_4$)$_2$SO$_4$, and 10 ml concentrated base (pH 6.8). The concentrated base (pH 6.8) contained (added to ddH$_2$O in the following order) 14.6 g KOH, 20 g nitriloacetic acid, 28.9 g MgSO$_4$ anhydrous, 6.67 g CaCl$_2$·7H$_2$O, 18.5 g (NH$_4$)$_6$MO$_7$O$_{24}$·7H$_2$O, 0.198 g FeSO$_4$·7H$_2$O, and 100 ml Metals 44. The Metals 44 contained (added to ddH$_2$O in the following order) 2.5 g EDTA (pH 8.0), 10.95 g ZnSO$_4$·7H$_2$O, 5 g FeSO$_4$·7H$_2$O, 1.54 g MnSO$_4$·H$_2$O, 0.392 g CuSO$_4$·5H$_2$O, 0.251 g CuSO$_4$ anhydrous, 0.250 g Co(NO$_3$)$_2$·6H$_2$O, adding a few drops of H$_2$SO$_4$ to avoid precipitation. Psmm must be sterilized in the same way as Ecmm above, with the salts (and metals) separate from the agar.

For the mating procedure P. aeruginosa strains were cultivated on Difco® Pseudomonas isolation agar (PIA), which contains Igrasan® to ensure the elimination of non-pseudomonads.

Pseudomonas broth (PB) was used to maximize pigment production in liquid culture for quantitative measurements. PB variations King’s A and B media (King et al. 1954) were used to study pyocyanin and pyoverdin production, respectively. King’s A broth contained 20 g Difco®
Bacto® peptone, 3 g MgCl₂ · 6 H₂O, 10 g anhydrous K₂SO₄, and 10 ml glycerol. King’s B broth contained 20 g Difco® proteose peptone No. 3, 1.5 g anhydrous K₂HPO₄, 1.5 g MgSO₄ · 7 H₂O, and 10 ml glycerol.

Peptone trypticase soy broth (PTSB) was the medium used to assay the production of the exoproducts casein protease and elastase. The medium contained 5% Difco® peptone and 0.25% Difco® trypticase soy broth (Diener et al. 1973).

Chrome azurol sulfate (CAS) medium (Schwyn and Neilands 1987) is a minimal medium used to study the iron chelating capacity of *P. aeruginosa* PAO1 and HD3β. This medium is difficult to make. The final color of the plates should be a vibrant, dark blue. A turquoise green color will be the result if the pH is above 6.6. CAS must also be sterilized in the same way as Ecmm above, with the salts separate from the agar and nutrients added after cooling. CAS contained 30.24 g PIPES, 6.0 g NaOH, 100 ml 10X MM9. The salts mixture pH was adjusted to 6.3 before autoclaving. TenX MM9 contained 60 g Na₂HPO₄, 3 g KH₂PO₄, 5 g NaCl, 10 g NH₄Cl. After sterilization the following components were added: 30 ml 10% deferrated casamino acids, 10 ml deferrated 20% succinate, 100 ml CAS-HDTMA, 1 mM MgSO₄, and 0.1 mM CaCl₂. The casamino acids and succinate were deferrated with an equal volume of 3% (w/v) 8-hydroxyquinoline in chloroform, then the 8-hydroxyquinoline was extracted with an equal volume of chloroform. The deferrated solutions were then autoclaved to sterilize. CAS-HDTMA is made up of two solutions, CAS and hexadecyltrimethylammonium bromide (HDTMA) mixed together to a total volume of one liter. The CAS solution contained (added to ddH₂O in the following order) 605 mg CAS and 100 ml iron (III) solution (1 mM FeCl₃ · 6 H₂O in 10 mM HCl). The HDTMA solution contained 729 mg HDTMA. The CAS solution was added to the HDTMA solution while stirring.
In the method of Brichta (2003), blood agar (BA) medium, supplied by BBL®, containing a tryptic soy agar (TSA) base and 5% sheep blood, was used to test the *in vitro* pathogenicity of *P. aeruginosa* PAO1, HD3β, and PAO1DB92.

Rhamnolipid plates were made in the method of Köhler et al. (2000). The final color of the plates should be a light, pastel blue. The medium must be sterilized in the same way as Ecmm above, with the salts separate from the agar and nutrients added after cooling. The medium was made of 0.0005% (w/v) methylene blue, 0.02% (w/v) cetyltrimethylammonium bromide, 0.05% (w/v) NaCl, 0.3% (w/v) KH₂PO₄, 0.6% (w/v) Na₂HPO₄, 2 mM MgSO₄, 100 ml 1X trace elements. After sterilization 0.05% glutamic acid and 0.2% glucose were added. OneX trace elements contained 23.2 mg H₃Bo₃, 17.4 mg ZnSO₄ · 7 H₂O, 23 mg FeNH₄ (SO₄)₂ · 12 H₂O, 9.6 mg CoSO₄ · 7 H₂O, 2.2 mg (NH₄)₆Mo₇O₂₄ · 4 H₂O, 0.8 mg CuSO₄ · 5 H₂O, and 0.8 mg MnSO₄ · 4 H₂O.

Four different media were used for the motility assays. Cross-streak inhibition assays used TSA plates (Brock et al. 1991), consisting of 40 g Difco® tryptic soy agar. Swim, swarm and twitch media were prepared in the method of Rashid and Kornberg (2000). Swim agar plates were 1% tryptone, 0.5% NaCl, and 0.3% agarose (in stead of the usual 1.6% agar). These plates were quite watery and were parafilmed closed to retain moisture before inoculation. Swarm agar plates were made of 8 g nutrient broth, 0.5% dextrose, and 0.5% agar (in stead of the usual 1.6% agar). Twitch agar plates were made of LB (10/5/5) broth and 1.0% agar (in stead of the usual 1.6% agar). The twitch plates were also poured much thinner than usual, using about 13 ml of medium and swirling the plate to get complete coverage.
Growth Conditions

Preparation of Cell Extracts for Enzyme Activity Assays

Cell extracts of PAO1 and HD3β were prepared from 50 ml cultures. Cells were collected by centrifugation (25 minutes at 1300×g at 4°C) during the late exponential phase (OD
\textsubscript{600} = 0.8 to 1.0) of growth. The cells were washed in Psmm salts only (without the added nutrients), collected by centrifugation as described above, and resuspended in 2 ml aspartate transcarbamoylase (ATCase) breaking buffer (2 mM β–mercaptoethanol (BME), 20 µM ZnSO
\textsubscript{4}, 50 mM tris-HCl (pH 8.0) and 20% glycerol). The suspension was subjected to ultrasonic disruption using a Branson cell disruptor 200 for 1 minute at 0°C, followed by 1 minute of rest at 0°C, repeated until the suspension was clear. While the cells were being broken, the 50 ml conical tube was secured in an ethanol-ice water slurry to control heating of the sample. The disrupted cell suspension was distributed into 1.5 ml centrifuge tubes and centrifuged at 9,000×g for 4 minutes. A 300 µl aliquot of this “slow spin” supernatant, containing a significant portion of the membrane components, was removed to a fresh tube and stored at 4°C. The remaining extract was centrifuged at 12,000×g for an additional 10 minutes at 4°C. Both the slow spin and regular cell extracts were then transferred to previously prepared dialysis tubing and dialyzed for 20 hours at 4°C in a 2 L plastic beaker with stirring. Dialysis tubing was prepared in the method of Sambrook et al. (1989). The dialysis buffer consisted of the same ingredients used to break the cells, except for the glycerol (50 mM tris-HCl (pH 8.0), 2 mM BME, and 20 µM ZnSO
\textsubscript{4}). The recommended dialysis buffer volume of 150 times the total cell extract volume was used. Each dialyzed cell extract was then transferred to a new centrifuge tube and stored at 4°C until assayed. Assays were performed within 48 hours of cell extract preparation.
For the pyrimidine starvation experiments the uracil-requiring, gentamicin-resistant mutant was grown in Psmm containing 40 µg/ml uracil and 100 µg/ml gentamicin to the late exponential phase of growth. The cells were used for a 1% inoculum of Psmm containing 5 µg/ml uracil and 100 µg/ml gentamicin. The OD<sub>600</sub> was measured each half hour until there was no further increase in OD<sub>600</sub>, at which point the cells were allowed to starve for 4 hours. The cells were then collected and slow spin and regular extracts were prepared as described above.

*Preparation for Pyocyanin Assays*

PAO1 strains were cultured in 5 ml of King’s A broth, with the appropriate uracil and gentamicin, incubated overnight at 37°C with shaking. Using the overnight culture, 30 ml of King’s A broth was inoculated with 1% of its volume in 125 ml Erlenmeyer flasks. Cultures were incubated at 37°C for 20 hours. One ml was removed from each culture and the OD<sub>600</sub> measured. The cultures were then immediately used for the assay.

*Preparation for Pyoverdin Assays*

PAO1 strains were cultured in 5 ml of King’s B broth, with the appropriate uracil and gentamicin, incubated overnight at 37°C with shaking. Using the overnight culture, 30 ml of King’s B broth was inoculated with 1% of its volume in 125 ml Erlenmeyer flasks. Cultures were incubated at 37°C for 30 hours with shaking. At 12 hours and 30 hours 1 ml was removed from each culture and the OD<sub>600</sub> measured. The cultures were then immediately used for the assay.

*Preparation of Supernatant for Elastase and Casein Protease Assays*

PAO1 strains were cultured in 5 ml of PTSB, with the appropriate uracil and gentamicin, incubated overnight at 37°C with shaking. Using the overnight culture, 10 ml of PTSB was inoculated with 1% of its volume in 125 ml Erlenmeyer flasks. Cultures were incubated for 24
hours at 37°C with shaking. One ml was removed from each culture and the OD$_{600}$ measured. The cultures were transferred to 15 ml conical tubes and centrifuged at 1300×g for 20 minutes at 4°C. The supernatant was filtered using 0.45 µm polysulfone membrane filters. One ml was removed from each filtered sample and the absorbance measured at 600 nm (A$_{600}$). The supernatant measurements were used as blanks and subtracted from the PTSB cell culture OD$_{600}$ values. The remainder of each filtered sample was stored at -80°C until the supernatant could be assayed.

Methods

Competent Cells

Competent cells were prepared in the method of Mandel and Higa (1992) to perform transformations. *E. coli* cells were grown in LB broth to OD$_{600}$ of 0.3 to 0.4. The cells were collected by centrifugation for 10 minutes at 1875×g at 4°C. The cells were washed in 20 ml 0.1 M CaCl$_2$ and collected as before by centrifugation. The cells were washed again in 20 ml 0.1 M CaCl$_2$ and allowed to incubate on ice for 30 minutes. The cells were collected as before by centrifugation. The cells were resuspended in 1 ml 0.1 M CaCl$_2$ and left overnight at 4°C with the conical tubes submerged in ice. On the following day sterile glycerol was added to the cells in CaCl$_2$ to a final concentration of 15%. The cells were then distributed into 100 µl portions and put into -80°C. After the aliquots were frozen solid they were ready for use and stable in -80°C storage for at least 4 months.

Transformations

Transformations were performed in the method of Sambrook et al. (1989). Competent cells (as prepared above) were allowed to thaw while on ice. Once thawed 100 to 500 µg transforming DNA was added to the cells. The cells were then incubated on ice for 30 minutes
and then heat shocked at 42°C for 60 seconds. One ml LB was added to the cells, which were then incubated at 37°C for 1 hour with shaking. The cells were collected by centrifugation (10 seconds at maximum speed in a microcentrifuge) and washed with 1 ml of Ecmm salts only (no added nutrients), or PBS. The cells were collected and washed again in the same way and 50 µl of the suspension was distributed to each plate for selection. The cells were collected as above, and all but 50 µl×N (where N = the number of desired plates) of the supernatant was poured off, and the cells were resuspended. Fifty µl of the concentrated suspension was distributed to each plate for selection.

**Ligations**

Ligations were performed in the method of Sambrook et al. (1989). All ligations performed were done using blunt-ended DNA. The DNA concentrations were mixed in a stoichiometric ratio of 1-vector : 2.6-insert. To lessen the occurrence of re-circularized vectors, the vector fragments were treated with New England Biolabs® (NEB) calf intestinal phosphatase to remove the 5' phosphate groups. The ligation buffer used was as specified in Sambrook et al. (1989) to increase the efficiency of blunt-end ligations. The reaction contained 6.25 mM polyethylene glycol (PEG) 8000, 50 mM tris (pH 7.6), 10 mM MgCl₂, 0.5 mM ATP (less than called for in a cohesive-end ligation), 1.5 µM hexamine cobalt chloride (HCC), 2 mM dithiothreitol (DTT) (1 M DTT = 309 mg DTT in 2 ml 0.01 M sodium acetate (pH 5.2), sterilized by filtration). Additionally, the units (U) of T4 DNA ligase were increased to 100 U per 3 µg DNA. The reactions were allowed to run anywhere from 4 to 16 hours at 20°C. On one occasion a successful ligation was performed using 2000 U high T4 DNA ligase (2000 U/µl) in a 10 minute reaction at room temperature.
Mating and Selection

PAO1 was grown in LB broth at 42°C with shaking to mid-log phase (OD$_{600} = 0.4$). Growing PAO1 at this elevated temperature facilitates mating. *E. coli* SM10 transformed with pHH08 was grown in LB broth at 37°C with shaking to mid-log phase (OD$_{600} = 0.4$). The PAO1 and SM10 cultures were combined in microcentrifuge tubes (MCTs) in the ratio of 20 SM10 : 1 PAO1, and also in the ratio of 10 SM10 : 1 PAO1. The mixed cultures were collected by centrifugation at 7,000 rpm in a Sorvall® microcentrifuge for 1 minute. The supernatant was poured off and the cells resuspended in 30 µl LB broth. The suspension was pipetted onto an LB plate in one spot and allowed to mate. Two mating plates were set up for each ratio combination, one for 5 hours of incubation at 37°C and one for 12 hours of incubation at 37°C. At the indicated time points the mating growth was scraped off of the agar and suspended in 1 ml of PBS. Dilutions were then made and 50 µl of $10^{-4}$ through $10^{-7}$ dilutions were spread plate onto PIA with Gm$_{100}$. The plates were incubated at 37°C for 12 hours. PIA with Gm$_{100}$ was used so that the *E. coli* SM10 would not survive, and so that any surviving PAO1 colonies would have to have pHH08 incorporated in its genome by a single recombination. These colonies were then picked and touched to two patch plates: 1) PIA with Gm$_{100}$ and 2) PIA with Cb$_{600}$, and allowed to incubate at 37°C for 12 hours. Colonies that grew on both plates were picked and grown in LB with Gm$_{100}$ another 12 hours at 37°C with shaking to allow time for a second recombination. The cells were collected by centrifugation and plated onto PIA with Gm$_{100}$. Colonies were picked with sterile toothpicks, touched to two patch plates: 1) PIA with Gm$_{100}$ and 2) PIA with Cb$_{600}$, and allowed to incubate at 37°C for 12 hours. Colonies that grew on 1) but not on 2) were then touched onto two more patch plates: 3) Psmm and 4) Psmm with U$_{40}$. The colonies that grew on 4) but not on 3) were the new *pyrB* mutant.
Protein Activity Gel

An ATCase protein activity gel was performed in the method of Gerhart and Pardee (1962), and visualized using non-denaturing polyacrylamide gel electrophoresis (PAGE). A non-denaturing gel was used because the full size of the protein was desired, with all of its subunits. Solutions A, B and C can be made ahead of time and stored at 4°C. Solution A: 1% bis, 30% acrylamide. Solution B: 1.5 M tris (pH 8.8). Solution C: 1 M tris (pH 6.8). Ten percent ammonium persulfate solution and 500 ml of 50 mM histidine buffer (pH 7.0) should be made fresh and put into 4°C. The vertical gel rig was set up and fitted with a comb in the gap at the top. A line was drawn 15 mm below the bottom of the comb teeth, and the comb removed. An 8% separating gel (4.83 ml ddH$_2$O, 2.67 ml solution A, 2.5 ml solution B, 50 µl 10% ammonium persulfate and 5 µl N,N,N',N'-tetramethylene-ethylene-diamine (TEMED) (a catalyst for ammonium persulfate polymerization)) was mixed and loaded into the vertical gel rig using a glass pipette. The cassette was filled up to the drawn line. One-butanol was loaded on top of the separating gel up to the top of the opening between the rig plates. This aids in polymerization by removing any air from the gel surface. After the gel hardened (15 to 30 minutes) the 1-butanol was drained off and the gel surface flushed with ddH$_2$O. The comb was reinserted and a 4% stacking gel (1.84 ml ddH$_2$O, 0.536 ml solution A, 0.800 ml solution C, 30 µl 10% ammonium persulfate, 5 µl TEMED) was prepared and loaded on top of the separating gel using a glass pipette. This gel hardens in about 45 seconds and must be loaded quickly. The running buffer (25 mM tris and 192 mM glycine) was mixed and poured into the gel rig middle and outside tanks just above the level of the gel. Cell extracts were prepared as above. Twenty µl cell extract was added to a MCT. Six µl 5X loading dye was added to each tube. The loading dye is a rust color, which turns blue on contact with proteins. The loading syringe was rinsed with
running buffer between the loading of each sample. The lid was placed on the gel rig, securing the positive and negative terminals to their power source. It was visually verified that the positive wire ran along the bottom of the tank and the negative wire ran along the top. The gel was run at 100 volts for 2 hours. Once the proteins in the samples were separated by size through electrophoresis, the gel was taken out of the rig and put through the following reaction to detect ATCase. The gel was incubated for 5 minutes in 250 ml of the cold 50 mM histidine solution with gentle rocking. Five ml of 1 M aspartate and 10 ml of 0.1 M carbamoylphosphate were prepared, added to the histidine buffer and the gel was incubated for another 5 minutes. This supplies the components necessary for the second reaction shown in Figure 1 a). The gel was then washed three times with ddH$_2$O and drained. Three mM lead nitrate was added to the remaining 250 ml of cold histidine buffer and poured over the gel. The gel was incubated at room temperature until a white precipitate (carbamoylaspartate, the product of the reaction carried out by ATCase) appeared in the gel (about 5 minutes). The gel was then washed three times with ddH$_2$O, soaked in 1% ammonium sulfide to intensify the staining, and rinsed with ddH$_2$O. The gel was wrapped in plastic wrap and scanned into a digital file.

**Southern Blot**

A Southern blot was performed using the procedure described in Sambrook et al. (1989) with slight modifications. The gel was 1% agarose stained with ethidium bromide. The membrane used was a Nalgene® positively charged nylon membrane. The membrane was always handled with sterile forceps to avoid foreign DNA contamination, and all paper described was handled with gloved hands. The solutions used were as prescribed without modification. The gel was electrophoresed with very low voltage to get better size separation. The gel was initially electrophoresed at 65 volts for 30 minutes to get the DNA out of the wells, then at 25
volts for 20 hours, and then again at 100 volts for 30 minutes to tighten up the bands. Once electrophoresed, a picture was taken to document the ladder location, and a series of washes were performed on the gel. Two 15 minute denaturizing washes with gentle rocking in 100 ml of 1X denaturation solution (1.5 M NaCl, 0.5 N NaOH). Three 30 minute neutralizing washes with gentle rocking in 100 ml of 1X neutralization solution (1 M tris (pH 7.4), 1.5 M NaCl). A gel stand wrapped with Whatman® paper was set into a baking dish and transfer solution was poured into the dish to fill half way up the gel stand and paper. The DNA denatured and neutralized gel was trimmed and notched on one corner for orientation recognition and placed wells down (DNA up) onto the stand wrapped with Whatman® paper soaked in transfer solution. The membrane was thoroughly wetted with sterile ddH$_2$O, placed onto the gel and marked with a permanent marker in one corner to indicate the non-DNA side, and the well location on the membrane. On top of the membrane, 3 pieces of Whatman® paper, a stack of paper towels, and a glass plate weight were used as a wick system to pull the transfer solution up through the gel and into the membrane. The transfer was allowed to proceed for 23.5 hours, with periodic exchange of wet paper towels for dry ones, and replenishment of transfer solution. The transfer solution used for this high percentage G/C content DNA was 20X SSC. The membrane was removed from the gel put DNA side up onto a piece of Whatman® paper wet with 10X SSC. The DNA was then fixed to the membrane using ultra violet radiation in a UVP CL-1000 ultraviolet crosslinker. The membrane was exposed for 40 seconds at 1500 x 100 µJ/cm$^2$, and then allowed to air dry on a dry paper towel. The membrane was sandwiched in Whatman® paper, wrapped in aluminum foil and stored at 4°C until ready for hybridization with probe.

Probe creation, their hybridization to the Southern blot, and their detection was achieved using the Roche® DIG High Prime DNA Labeling and Detection Starter Kit II, and the
accompanying DIG Wash and Block Buffer Set. The procedure described in the Roche® kit was conducted without modification. First the probes were engineered, created and tested. Then the membrane was hybridized with the DNA probes which were randomly labeled with digoxigenin-dUTPs. Once hybridized the probes are immunodetected with the antibody anti-digoxigenin-AP (AP standing for alkaline phosphatase). The probes can then be visualized with the chemiluminescence substrate CSPD®, ready-to-use (kit supplied formulation of the substrate in an alkaline buffer solution). Enzymatic dephosphorylation of CSPD® by AP leads to light emission at 477 nm. Since the probe now identified with antibody is the only source of alkaline phosphatase, the CSPD® will only emit light where the probe is. The light emission continues for up to 48 hours after applying the CSPD®, ready-to-use.

The probes were hybridized to the membranes in separate hybridization canisters in a hybridization oven for 20 hours at 52°C. The probed membranes were washed with high stringency twice in 2X SSC, 0.1% SDS at room temperature for 5 minutes, and twice in 0.5X SSC, 0.1% SDS at 68°C for 15 minutes. The membranes were washed in washing buffer, blocking solution, antibody solution and treated with alkaline phosphatase detection buffer according to the DIG High Prime procedure. The membrane was then treated with 1 ml of CSPD®, ready-to-use and covered quickly with a plastic developing folder to spread the CSPD®, ready-to-use over the membranes via capillary action. The treated membranes were secured DNA side up in a metal developing folder and in a dark room X-ray film was laid down on top of the membranes and the folder clamped shut. The film was exposed to the chemiluminescence for 10 minutes and subsequently developed in an automatic film developer machine.
Growth Characteristics

There were two procedural methods learned very late in this study both involving spectrophotometrical measurements of samples. The first method involves the magnitude of optical density (OD) measurements. The measurements of cells in culture made by the spectrophotometer become increasingly less accurate as they increase above a value of 1.00. Therefore, it is necessary to make dilutions of the test specimen as necessary to achieve an OD reading below 1.00, and then adjust the OD value back up by that dilution factor. This was not done in any of the measurements to follow in the results of this study. The second method involves the practice of subtracting off the background value that exists because of the liquid in which the cells are being measured. It is necessary to subtract off the OD of not only the medium, but in the case of *P. aeruginosa* also the exoproducts that may or may not have been produced as well. This is accomplished by centrifugation of the measured cell culture sample, and again measuring the OD of the filtered supernatant. The supernatant OD is then used as the background value for that particular sample. This adds an order of magnitude increase in workload, because one blank value consisting of the medium OD cannot be used for all samples. Each culture has its own individual level of exoproduct production. The procedures below all used the growth medium alone for the background OD value, except for as described in the elastase and casein protease assays below.

*Pyrimidine Biosynthetic Pathway Protein Activity Analysis*

Cell extracts for the following assays were prepared as described above. Three independently grown samples were prepared for each organism. Additionally, each independent sample was assayed in triplicate and averaged for an enzyme activity value. The mean and
standard deviation of the averaged values were calculated and reported for that organism at that particular growth condition.

Protein concentration of each cell extract was estimated using the BioRad® Bradford protein detection assay (Bradford 1976). Once assayed, measurements were compared to a lysozyme standard curve to estimate an equivalent protein concentration existing in the cell-free extract. The standard curve was constructed with the same Bradford protein detection assay, using known quantities of lysozyme ranging in concentration from 0 to 20 µg/ml, incubated at the same temperature and for the same amount of time.

**Aspartate transcarbamoylase (ATCase pyrBC') assay.**

ATCase activity was measured by quantifying the amount of carbamoylaspartate (CAA) produced in 20 minutes at 30ºC by conversion from carbamoylphosphate (CP) and aspartate (Fig. 1a). This was accomplished using the method of Gerhart and Pardee (1962), with modification, using the color development procedure of Prescott and Jones (1969). A 1 ml reaction volume contained 4% tribuffer (0.051 M diethanolamine, 0.051 M N-ethylmorpholine, and 0.1 M MES (filter sterilized), adjust pH to 9.5 with KOH), 20 mM-L-aspartate-monopotassium, 5 mM CP-dilithium salt, and cell extract (the concentration of which depended on the initial estimate of protein content as described above). Tubes containing buffer and cell-free extract were prepared and incubated at 30ºC for 5 minutes. The reaction was initiated with the addition of CP (which was a fresh stock of 50 mM CP). Assay tubes were incubated at 30ºC for an additional 20 minutes. The reaction was stopped by the addition of 1 ml of color mix, comprised of 1 part monoxime (0.8 g monoxime in 100 ml 5% acetic acid) and 2 parts antipyrine (0.5 g antipyrine in 100 ml 50% H₂SO₄). The tubes were vortexed, capped with marbles and incubated uncovered (exposed to normal laboratory lighting) at 65ºC for 50 minutes. The $A_{466}$
of each assay tube was measured. The amount of CAA produced was determined using a CAA standard curve. The standard curve was prepared using the same buffering system and known concentrations of CAA, ranging from 0 to 0.6 mM, in the standard assay mix incubated at the same temperature and for the same amount of time.

Dihydroorotase (DHOase \(pyrC\)) assay.

DHOase activity was measured using the reverse assay of Beckwith et al. (1962), by quantifying the amount of CAA produced by conversion from dihydroorotate (DHO) (Fig. 1a). A 1 ml reaction volume contained 100 mM tris-HCl buffer (pH 8.6), 1 mM EDTA, 2 mM-L-DHO, and cell extract. Tubes containing buffer and cell-free extract were prepared and incubated at 30°C for 5 minutes. The reaction was initiated with the addition of DHO (which was a fresh stock of 20 mM DHO in 0.1M KPB). Assay tubes were incubated at 30°C for an additional 20 minutes. The reaction was stopped by the addition of 1 ml of color mix (as above). The tubes were vortexed, capped with marbles and incubated uncovered (exposed to normal laboratory lighting) at 65°C for 50 minutes. The \(A_{466}\) of each assay tube was measured. The amount of DHO converted to CAA was determined with a CAA standard curve as above in the ATCase assay.

Dihydroorotate dehydrogenase (DHOdehase \(pyrD\)) assay.

DHOdehase activity was measured spectrophotometrically at 290 nm by monitoring the oxidation of DHO to orotate (Beckwith et al. 1962; Schwartz and Neuhard 1975) (Fig. 1b). The reaction volume was 1 ml and was prepared in a quartz cuvette. The reaction mix contained 100 mM tris-HCl buffer (pH 8.6), 6 mM MgCl\(_2\), 1 mM L-DHO and the “slow spin” fraction of cell extract. Cuvettes containing buffer and cell-free extract were prepared and incubated at 30°C for 5 minutes. The reaction was initiated with the addition of DHO (which was a fresh stock of 20
mM DHO in 0.1 M KPB). The $A_{290}$ was measured every 5 minutes for 15 minutes. An increase in the $A_{290}$ of the reaction mix by 1.93 is equivalent to a change in substrate concentration of 1 mM.

**Orotate phosphoribosyltransferase (OPRTase pyrE) assay.**

OPRTase activity was measured spectrophotometrically at 295 nm by monitoring the conversion of orotate to orotidine 5'-monophosphate (OMP) (Beckwith et al. 1962; Schwartz and Neuhard 1975) (Fig. 1b). The 1 ml reaction volume was prepared in a quartz cuvette. The reaction mix contained 100 mM tris-HCl buffer (pH 8.6), 6 mM MgCl$_2$, 0.25 mM orotate, 0.6 mM 5-phosphoribosyl-1-pyrophosphate (PRPP) and cell extract. Cuvettes containing buffer and cell-free extract were prepared and incubated at 30°C for 5 minutes. The reaction was initiated with the addition of PRPP (frozen stock). The $A_{295}$ was measured every 5 minutes for 15 minutes. A decrease in the $A_{295}$ of the reaction mix by 3.67 is equivalent to an increase in OMP concentration of 1 mM.

**Orotidylate decarboxylase (OMPdecase pyrF) assay.**

OMPdecase activity was measured spectrophotometrically at 285 nm by measuring the conversion of OMP to uridylate (UMP) (Kelln et al. 1975; Abdelal et al. 1977) (Fig. 1b). The reaction volume was 1 ml and was prepared in a quartz cuvette. The reaction mix contained 100 mM tris-HCl buffer (pH 8.6), 6 mM MgCl$_2$, 0.2 mM OMP and cell extract. Cuvettes containing buffer and cell-free extract were prepared and incubated at 30°C for 5 minutes. The reaction was initiated with the addition of OMP (frozen stock). The $A_{285}$ was measured every 5 minutes for 15 minutes. A decrease in the $A_{285}$ of the reaction mix by 1.38 is equivalent to a decrease in OMP concentration of 1 mM (Beckwith et al. 1962; Schwartz and Neuhard 1975).
Ornithine transcarbamoylase (OTCase $argF$) assay.

OTCase activity was measured by quantifying the amount of citrulline produced by conversion from CP and ornithine (Kelln et al. 1975; Abdelal et al. 1977) (Fig. 4). A 1 ml reaction volume contained 100 mM tris-HCl buffer (pH 8.6), 4 mM CP, and 10 mM-L-ornithine and cell extract. Tubes containing buffer and cell-free extract were prepared and incubated at 30°C for 5 minutes. The reaction was initiated with the addition of CP. Assay tubes were incubated at 30°C for an additional 10 and 20 minutes. The reaction was stopped by the addition of an equal volume of color mix (as in ATCase assay above). The tubes were vortexed, capped with marbles and incubated uncovered (exposed to normal laboratory lighting) at 65°C for 50 minutes. The A$_{466}$ of each assay tube was measured. The amount of citrulline produced was determined using a citrulline standard curve. The standard curve was prepared using the same buffering system and known concentrations of citrulline, ranging from 0 to 0.5 mM, in the standard assay mix incubated at the same temperature and for the same amount of time.

**Virulence Analysis**

Media, cultures and supernatants were prepared for the following assays as described above. Three independently grown samples were prepared for each organism. Additionally, each independent sample was assayed in triplicate and averaged. The mean and standard deviation of the averaged values were calculated and reported for that organism at that particular growth condition.

**Exoproducts.**

**Pyocyanin assay.**

Pyocyanin production was quantified using the method of Essar et al. 1990. The assay is based on the absorbance of pyocyanin at 520 nm in acidic solution (Kurachi 1958; MacDonald
PAO1 strains were cultured in King’s A medium as described above. Cells from 5 ml of culture were pelleted by centrifugation at 1300×g for 25 minutes at 4°C. Pyocyanin was extracted from the supernatant by the addition of 3 ml of chloroform. The pyocyanin was extracted from the chloroform by the addition of 1 ml of 0.2 N HCl. The resulting pink to deep red HCl solution (top layer) was transferred to a 1.5 ml centrifuge tube and the $A_{520}$ was measured (in a quartz cuvette). Concentrations, expressed as micrograms of pyocyanin produced per 5 ml of culture supernatant, were determined by multiplying the $A_{520}$ by the Kurachi factor of 17.072 (Kurachi 1958).

*Pyoverdin assay.*

Pyoverdin was measured as a function of bacterial growth in King’s B medium. At 12 hours and 30 hours 1.5 ml was removed from each culture. The $OD_{600}$ was measured and the cells pelleted by centrifugation at 10,000 rpm in a Sorvall® microcentrifuge for 5 minutes at 4°C. The absorbance of the supernatant was measured at 405 nm. Pyoverdin levels were expressed as the ratio of $A_{405/OD_{600}}$ (Stintzi et al. 2000).

*Elastolysis assay.*

Elastolytic activity in PAO1 culture supernatants was determined by the elastin Congo red (ECR) assay as described by Pearson et al. (1997) with slight modification. Supernatants were prepared as described above. Twenty five µl samples of thawed supernatant were added to tubes containing 20 mg of ECR (Sigma®) in 1.3 ml of buffer (0.1 M tris (pH 7.2), 1 mM CaCl$_2$). Tubes were incubated for 18 hours at 37°C with shaking. Each reaction tube was incubated on ice for 10 minutes after the addition of 0.13 ml of 0.12 mM EDTA. Insoluble ECR was removed by centrifugation, and the $A_{495}$ of the supernatant was determined. Absorption due to pigments produced by PAO1 was corrected for by subtracting the $A_{495}$ of each sample that had been
incubated in the absence of ECR, plus the $A_{495}$ of buffer and ECR incubated in the absence of sample.

*Casein protease assay.*

Proteolytic activity was determined by an azocasein assay described by Kessler et al. (1993) with slight modification. Fifty µl of thawed supernatant, prepared as above, was added to 1 ml of buffer (0.05 M tris (pH 7.5), 0.5 mM CaCl$_2$) containing 0.3% azocasein (Sigma®). The tubes were incubated at 37°C for 15 minutes with shaking and then the reaction stopped by adding 0.5 ml of 10% (w/v) trichloroacetic acid. The samples were then centrifuged and the absorbance of the clarified supernatants was measured at 400 nm. Absorption due to pigments produced by PAO1 was corrected for by subtracting the $A_{400}$ of each sample that had been incubated in the absence of azocasein, plus the $A_{400}$ of buffer and azocasein incubated in the absence of sample.

*Rhamnolipid production assay.*

Test organisms PAO1, HD3β, and PAO1DB92 were streaked onto the rhamnolipid plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile toothpick and touched to the corresponding (with or without uracil) rhamnolipid plates. The plates were incubated at 37°C for 24 hours and then at room temperature (on the bench top) for another 24 hours. The pink rhamnolipid halo diameter and the diameter of the bacteria growth were measured and recorded.

Iron chelation assay.

Test organisms PAO1, HD3β, and PAO1DB92 were streaked onto the CAS plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile toothpick and touched to the corresponding CAS plates. The plates were incubated for 24 hours
at 37°C. At 18 hours and 24 hours the light salmon color halo diameter and the diameter of the bacteria growth were measured and recorded.

Haemolysin assay.

Test organisms PAO1, HD3β, and PAO1DB92 were streaked onto TSA plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile toothpick and touched to the corresponding BA plates. The BA plates were incubated for 48 hours at 37°C. At 24 hours and 48 hours the haemolyzed blood diameter and the diameter of the bacteria growth were measured and recorded.

Motility.

Cross-streak inhibition assay.

Test organisms PAO1, HD3β, and PAO1DB92 and cross-streak organisms *B. cepacia*, *E. coli*, and *S. aureus* were streaked onto TSA plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile loop and streaked onto TSA plates. First a line of one of the test organisms was put onto a plate near to one edge. The plates were incubated at 37°C for 18 hours to allow the synthesis of exoproducts. Next, a line was drawn on the bottom surface of the plate 5 mm away from, and parallel to the test organism growth. Each of the cross-streak organisms were then put in a line on the TSA plate starting at the 5 mm indicator line and continuing perpendicular to the test organism growth. The plates were then incubated at 37°C for 24 hours. Measurements were recorded for the distance between the test organism growth and cross-streaked organism growth. Distance measurements were also recorded for any apparent lack of normal growth, or inhibition.
Swimming assay.

Test organisms PAO1, HD3β, and PAO1DB92 were streaked onto LB plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile toothpick and touched to the corresponding swim plates. The plates were incubated at 37°C for 14 hours. Pictures were taken to document the resulting characteristics.

Swarming assay.

Test organisms PAO1, HD3β, and PAO1DB92 were streaked onto LB plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile toothpick and touched to the corresponding swarm plates. The plates were incubated at 30°C for 14 hours. Pictures were taken to document the resulting characteristics.

Twitching assay.

Test organisms PAO1, HD3β, and PAO1DB92 were streaked onto LB plates and incubated overnight at 37°C. Colonies were picked from the overnight culture with a sterile toothpick to inoculate the corresponding swarm plates. The toothpick was pressed through the agar surface and touched to the plastic plate underneath. The plates were incubated at 37°C for 24 hours. Measurements were taken for the diameter of the twitch halo, which had developed at the agar and plate interface. Phenotypes were also recorded.
CHAPTER III

RESULTS

Creating the pyrBC′ Mutant

The recombinant plasmids used to create the mutated pyrBC′ were successfully created and the mutated gene cloned into the broad-range-host vector pRTP1. Figure 11 is a picture of a 1% agarose gel electrophoresis of the two pieces used to ligate pH02. Lanes 1 through 3 show pGmΩ1 digested with SmaI. The 1.7-kbp pieces contain the gentamicin cassette. Lane 4 contains New England Biolabs® (NEB) 1kb ladder. Lanes 5 through 8 show pH01 digested with NcoI and EcoRV. Difficult to see, but directly beneath the dark color of the loading buffer is the 417-bp piece cut out of pyrB. The gentamicin cassette was ligated in place of the 417-bp piece with the 3875-bp large fragment of digested pH01 to form pH02. Figure 12 is a picture of a 1% agarose gel electrophoresis of the two pieces used to ligate pH08. Lane 1 contains NEB 1kb ladder. Lanes 2 through 4 contain pH02 digested with SacI and SphI. The larger 2.95-kbp band contains the mutated pyrBC′ with the inserted gentamicin cassette. Lane 5 contains uncut pH02. Lane 6 contains pRTP1 digested with EcoRI, blunted with the Klenow fragment and end phosphate groups removed with calf intestinal phosphatase. The prepared pRTP1 vector was ligated with the 2.9-kbp piece of pH02 to form pH08. Figure 13 is a picture of a 1% agarose gel electrophoresis of digested pH08. These digests were performed to verify the plasmid’s size and genetic makeup. Lane 1 contains NEB 1kb ladder. Lane 2 contains pH08 digested with HindIII. It was expected that HindIII would cut pH08 3 times; twice in the multiple cloning sites around the gentamicin cassette fragment (Fig. 14), yielding the 1.7-kbp cassette, and once in the pRTP1 vector (Fig. 15). However the HindIII cut site in pRTP1 is so
Figure 11. pHH01 and pGMΩ1 Restriction Digests
Figure 12. pHH02 and pRTP1 Restriction Digests
Figure 13. pHH08 Restriction Digest
Figure 14. Plasmid Map of pGM\(\Omega\)1
Figure 15. Plasmid Map of pRTP1
close to the EcoRI insert site of the mutated \textit{pyrBC'}, that it would yield only the portion of \textit{pyrB} upstream of the gentamicin cassette (about 600 bp), and the rest of the vector plus the portion of downstream of the gentamicin cassette in the other band (Fig. 9). Therefore, the three band sizes predicted were 600-bp, 1.7-kbp, and 8.2-kbp. Employing the technique of preparing the gel with ethidium bromide in the solution before electrophoresis, and not yet knowing that one could also put the electrophoresed gel into an ethidium bromide bath to intensify the DNA visibility, resulted in a 600-bp band is barely visible. However, the resulting bands were just as expected. Lane 3 in Figure 13 contains pHH08 digested with \textit{SmaI}. Lane 4 contains uncut pHH08. Lane 5 contains pUC18 digested with \textit{SmaI}. It was expected that \textit{SmaI} would cut pHH08 three times; once in the pUC19 polylinker sequence on the downstream side of the mutated \textit{pyrBC'} (Fig. 9), and twice in pRTP1 (Fig. 15). The resulting banding pattern of this digest would indicate the direction in which the mutated gene had cloned into the vector. A pattern of 2.7-kbp containing \textit{ori}, 3.4-kbp containing mutated \textit{pyrBC'} and 4.4-kbp containing ampicillin resistance (Amp'), would mean that the segment had been cloned in the same direction as the Amp' gene, because the \textit{SmaI} site of the mutated insert would be on the opposite side of the mutated gene from the pRTP1 \textit{SmaI} site near \textit{ori}. Alternatively, a banding pattern of 1.0-kbp containing the pRTP1 \textit{HindIII} and \textit{BamHI} sites, 2.7-kbp containing \textit{ori} and 6.8-kbp containing mutated \textit{pyrBC'} and Amp' would indicate that the segment had been cloned in the opposite direction to the Amp' gene (as pictured in Fig 9). Either orientation would be functional, because \textit{pyrBC'} has its own promoter in the upstream pUC19 \textit{lacZa} sequence. The resulting banding pattern in Figure 13 lane 3 is 2.7-kbp, 3.4-kbp and 4.4-kbp, indicating that the mutated \textit{pyrBC'} is in the same direction as the Amp' gene.

pHH08 was successfully transformed into with \textit{E. coli} SM10, which was mated with PAO1. After two unsuccessful mating attempts it was discovered that the SM10 strain was bad.
It grew initially, but then died off in stead of maintaining a stationary phase. New SM10 was grown from reliable freezer stock and verified for viability. The fresh SM10 was transformed with pHH08. This time the mating was successful on the first try. The screenings successfully identified a colony whose single recombination phenotype was gentamicin resistance and carbenicillin resistance, and whose double recombination phenotype was gentamicin resistance and carbenicillin sensitivity. This colony was then streaked onto a Pseudomonas minimal medium (Psmm) plate with uracil and Gm, and a Psmm plate with Gm and no uracil. Only the bacteria on the plate with uracil grew, verifying that the new organism was auxotrophic.

Verification of the pyrBC' Mutant

ATCase Protein Activity Gel

Results for the aspartate transcarbamoylase (ATCase) protein activity gel are shown in Figure 16. Any protein in the sample will show up as an ammonium sulfide stained band. Lane 1 contains a positive control sample from E. coli cell extract. One 309-kDa band is visible. Three bands were expected for E. coli which has a class B ATCase (Fig. 5). There should have been 3 bands, one at 309-kDa, 270-kDa and 100-kDa. The presence of only one band was attributed to the fact that the cell extract was old. Lane 2 contains the positive control sample from PAO1 cell extract. One 486-kDa band was visible. This was just as expected for P. aeruginosa which has a class A ATCase (Fig. 5). Lane 3 contains dialyzed HD3β cell extract. Lane 4 contains non-dialyzed HD3β cell extract. This was done to verify that there was no alteration in the protein make-up of the cell extract with dialysis. Both lanes show no protein banding, verifying that there is no ATCase in HD3β cells.
Figure 16. ATCase Protein Activity non-denaturing PAGE

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>E. coli</td>
</tr>
<tr>
<td>2</td>
<td>PAO1</td>
</tr>
<tr>
<td>3</td>
<td>HD3β – dialyzed</td>
</tr>
<tr>
<td>4</td>
<td>HD3β – not dialyzed</td>
</tr>
</tbody>
</table>

Figure 17. PAO1 and HD3β Genomic Digests for Southern Blot. This is a picture of a 1% agarose gel stained with ethidium bromide.
Southern Blot

Results of the digest performed on genomic PAO1 and HD3β are shown in Figure 17. The 1% agarose gel was set up symmetrically, so that the blotted membrane could be cut down the middle (leaving half a lane of ladder on each membrane) and the identical digests could then be hybridized by separate probes. Lanes 1/6 contain PAO1 digested with Smal. Lanes 2/7 contain HD3β digested with Smal. Lanes 3/8 contain PAO1 digested with AgeI. Lanes 4/9 contain HD3β digested with AgeI. Lane 5 contains NEB 1kb ladder. Smal was chosen because it would cut the PAO1 and HD3β genomes around pyrBC', such that pyrBC' would be contained entirely within a 3688-bp and 3066-bp piece, respectively. The HD3β piece is smaller, because the net change of the deletions from pyrBC', and the addition of the 1.7-kbp gentamicin cassette is a negative 622 bp. AgeI was chosen because it would cut the PAO1 genome in the middle of pyrBC', such that pyrBC' is contained in two fragments of 3390-bp and 2144-bp in length. Conversely, AgeI would not cut the HD3β genome in the middle of pyrBC', because that restriction site was contained in the 417-bp deletion from pyrB. Additionally, there are no AgeI sites within the gentamicin cassette, therefore pyrBC' would be contained entirely within one band of 4912-bp in length (3990 bp + 2144 bp – 622 bp = 4912 bp). The AgeI digest results in the optimum banding pattern, because it gives two distinct patterns and verifies the deletion in pyrBC'. These are the bands that should be hybridized by any probe that contains pyrBC' sequence. Figure 18 is a picture of the genomic digest with the predicted band locations located for each lane. The right side of the gel is an exact repeat of the left side, it would only be probed differently.

Each restriction digest resulted in a long smear of DNA fragments. It was not known if the smear was a good digest, or if a distinct banding pattern should have been observed (as seen when using other restriction enzymes in a genomic digest). However, the experiment was
Figure 18. Predicted Banding Pattern for Probed Southern Blot of PAO1 and HD3β Genomic Digests
continued as planned. This is precisely why two separate restriction enzymes were used. If SmaI didn’t cut the genomes as predicted, then AgeI would or vice versa. The Southern blot procedure was successfully completed and the membrane with the transferred DNA was cut down the middle of lane 5 in preparation for hybridization with the two probes.

Probe A10 was used to hybridize the left half of the membrane. A10 was created using the plasmid pA10 (Fig. 8). pA10 contains pyrBC’ completely in tact. The plasmid was digested with PstI, resulting in a 2508-bp fragment containing pyrBC’ and 236 bp downstream of pyrC’. The fragment was used as a template in an amplification reaction to create randomly formed oligonucleotides in which every dUTP is labeled with digoxigenin. Therefore the probe A10 consists of small, random, labeled fragments of all portions of its template sequence. What does this mean for the two digested genomes? A10 has oligonucleotides that will anneal with both the wild-type gene and the mutated gene, so all bands containing those annealed oligonucleotides should fluoresce when washed with antibody developer.

Probe HH02 was used to hybridize the right half of the membrane. HH02 was created using the plasmid pHH02 (Fig. 9). pHH02 contains the fully mutated form of pyrBC’. The plasmid was digested with SacI and SphI, resulting in a 2950-bp fragment containing the beginning of pyrB, the gentamicin cassette, the end of pyrB and beginning of pyrC’. The fragment was used as a template in an amplification reaction to create randomly formed oligonucleotides in which every dUTP is labeled with digoxigenin. Therefore the probe HH02 consists of small, random, labeled fragments of all portions of its template sequence. HH02 contains enough of the original gene to hybridize to the wild-type gene. This probe was created to show whether or not the gentamicin cassette was incorporated anywhere else in the HD3β genome. HH02 is the ideal probe, but not knowing how including the gentamicin cassette sequence might effect the hybridization, probe A10 was created as a backup.
As previously mentioned, the two separate probes should result in identical banding patterns via two different probe designs. Figure 19 shows the result of the hybridized and developed membranes. A10 probed membrane is on the right and the HH02 probed membrane is on the left. The hybridized DNA is in the predicted location for every digest, and both probes resulted in the same banding pattern. Unexpected was the hybridization of the NEB 1kb ladder. This is addressed further in the Discussion section in Chapter IV.

Characterization of the pyrBC’ Mutant

Growth Characteristics

The new pyrB mutant organism, named HD3β, required exogenous uracil in order to grow. Healthy cells grown in a uracil rich medium transferred to a minimal medium without uracil did not survive. This result indicates that the pyrBC’ was successfully knocked out. The initial growth of the mutant also demonstrated an obvious impaired ability to produce pigments, pointing to potentially decreased virulence. Also evident was the impaired growth rate of HD3β when grown in liquid medium. Figure 20 is a plot of growth curves for PAO1 and HD3β in Psmm. Each curve is the resulting average of three independent growth curves. Curve a) is PAO1 grown in Psmm curve and b) is PAO1 grown in Psmm + 40 µg/ml uracil. When uracil is added to the growth medium the salvage pathway is activated, and it takes 1.5 hours longer for the wild-type cells to reach the their maximum optical density (OD). In any minimal medium HD3β must be grown with added uracil, as demonstrated by the lack of growth shown in curve f), which is HD3β in Psmm alone. Curve c) is HD3β in Psmm + 40 µg/ml uracil, which shows a lag in growth as compared to PAO1. Figure 21 shows a comparison of the two growth curves. It takes 15 hours of growth for HD3β to reach the maximum OD, as compared to 9 hours for PAO1. Of note here is that HD3β does reach the same maximum OD as the wild type.
Figure 19. DIG-dUTP Hybridized Southern Blot of PAO1 and HD3β Genomic Digests.
Figure 20. PAO1 and HD3β Growth in *Pseudomonas* minimal medium
Figure 21. PAO1 and HD3β Growth in *Pseudomonas* Minimal Medium with 40 µg Uracil ml⁻¹
Figure 20 curve d) shows HD3β grown initially in Psmm + 40 µg/ml uracil, then moved as a 1% inoculum to Psmm + 5 µg/ml where growth continued, at an even further decreased rate until it ran out of exogenous uracil and started to starve. The last datum point on curve d) is at 4 hours of starvation. Curve e) is HD3β growing at an even further decreased rate, because it is in Psmm + 40 µg/ml orotate. Interestingly, the color of the growth varied with the organism and nutrients. The wild type grew with a dark, rich blue/green color. The mutant in Psmm with uracil grew with a light mint (retro) green color. The mutant in orotate grew with a creamy lemonade color.

Growth curves conducted in peptone trypticase soy broth (PTSB) are presented in Figure 22. Again there is a lag in the growth of HD3β, but it does also reach the PAO1 maximum OD. It takes 32 hours for HD3β to reach the maximum OD, as compared to about 16 hours for PAO1. As no data were taken between 14 and 24 hours, the 16 hour time estimation for PAO1 was extrapolated from the slope of the curve for data taken between 6.5 and 14 hours. The same was done for HD3β and HD3β grown with 0.05% Triton-X added. Triton-X was added to one of the HD3β samples, because the growth was so clumpy that the OD could not be accurately measured. This seemed to also effect the overall growth, as compared to HD3β in PTSB alone. The sample with Triton-X stayed in the log growth phase longer and then continued growth along the same shallow increase as HD3β in PTSB alone to the maximum OD. The estimated time for HD3β with Triton-X to reach maximum OD was 20 hours. The wild type compared to the mutant had a higher slope in the log phase, and then a lower slope in the shallow increase to its maximum OD. Figures 23 a) through d) show the data for the viable cell numbers (VCN) associated with the growth curves in Figure 22. When the VCN and OD are plotted together on a log scale, as in Figures 23 a) through c) it can be seen that the trend of the VCN data follows the OD measurement. For PAO1 and HD3β with Triton-X there is an evident drop in VCN at
Figure 22. Growth Curves in PTSB for PAO1, HD3β, and HD3β with 0.05% Triton-X
Figure 23 a) PAO1 Growth

Figure 23 b) HD3β Growth with 0.05% Triton-X

Figure 23. Growth in PTSB Medium vs. Viable Cell Number
Figure 23 c) HD3β Growth

Figure 23 d) Comparison of PAO1, HD3β with Triton-X, and HD3β VCN

Figure 23. Growth in PTSB Medium vs. Viable Cell Number
the late stationary phase. The same drop-off would likely be seen for HD3β without Triton-X had it been grown for another 5 to 10 hours.

It was noted in day to day procedures that when using established growth on Psmm plates to inoculate PTSB plates the PAO1 pyrimidine mutants had a more difficult time growing on the richer medium than they would have had they been established on that rich medium to start with. This was also observed when switching to Luria-Bertani (LB), *Pseudomonas* isolation agar (PIA) and *Pseudomonas* broth (PB) media. It is hypothesized in our laboratory that this may have something to do with the way the organism has to process the nutrients. PAO1 did not grow well when switched from Psmm to a richer medium, and HD3β grew even slower. HD3β did not produce any pigment in the first 24 hours of growth on PIA. It is hypothesized that with the shift in energy demand, the organism cannot spend its resources on the production of exoproteins.

**Pyrimidine Biosynthetic Pathway Protein Activity Analysis**

In order to reduce the data for protein activity, it was necessary to generate some standard curves. For each of the enzyme activity assays below there had to be an estimation of the initial protein content existing in the prepared cell extract to be analyzed. This was accomplished by measuring known quantities of the protein lysozyme, and comparing the unknown cell extract OD value to the lysozyme curve OD values. In this way an estimated protein quantity could be calculated from the equation of the curve. The lysozyme standard curve is presented in Figure 24. Similarly, a carbamoylaspartate (CAA) standard curve (Figure 25) was required for the analysis of ATCase and dihydroorotase (DHOase) activity, but in this case on the other end of the equation for the resulting protein created by the active enzyme. Similarly, a citrulline standard curve (Figure 26) was required for the analysis of ornithine transcarbamoylase (OTCase) activity, based on the production of citrulline. In each case, an OD value was known
Figure 24. Lysozyme Standard Curve

Figure 25. CAA Standard Curve

Figure 26. Citrulline Standard Curve
and a protein concentration was calculated using the equation of the standard curve.

Pyrimidine biosynthetic pathway intermediate enzyme activities are presented for PAO1 and HD3β in Figures 27 and 28, respectively. PAO1 supplied with exogenous uracil shows a reduction in all of the enzyme activities. Most significantly, the ATCase activity was reduced by 50%. Figure 28 shows that the pyrBC' mutant without being fed uracil has no ATCase, DHOase, or dihydroorotate dehydrogenase (DHOdehase) activity. The pathway is shut down by the inability to produce the intermediate CAA. When the mutant is fed uracil, results show that ATCase, DHOase, and DHOdehase are still shut down, but there is activity for orotate phosphoribosyl transferase (OPRTase) and orotidylate decarboxylase (OMPdecase). It seems that there is another way to the second half of the biosynthetic pathway. Figure 29 and 30 are comparisons of PAO1 and HD3β in Psmm with and without uracil, respectively. Figure 29 shows enzyme activity when both organisms have the salvage pathway activated. Most impressive is the complete shut down of the first half of the pathway for the mutant. Then remarkably the second half of the pathway shows the mutant enzyme activity near wild-type levels. Figure 29 also shows that there was increased OTCase activity in the mutant compared to the wild type when exogenous uracil was supplied. This suggests that there is excess carbamoylphosphate available and the arginine pathway is up-regulated to produce more arginine. Figure 30 shows the shut down of the pathway for the starving mutant.

Virulence Analysis

Exoproducts.

Pyocyanin and pyoverdin production.

Results for pyocyanin production are presented in Figure 31. Samples were incubated at 37°C with shaking for 20 hours in King’s A medium. The pyocyanin production is greatly reduced in the pyrBC' mutant. Expressed in percent of wild type, the pyocyanin production
Figure 27. Pyrimidine Biosynthetic Intermediate Enzyme Activities of PAO1

Figure 28. Pyrimidine Biosynthetic Intermediate Enzyme Activities of HD3β
Figure 29. Pyrimidine Biosynthetic Intermediate Enzyme Activities of PAO1 and HD3β with Exogenous Uracil

Figure 30. Pyrimidine Biosynthetic Intermediate Enzyme Activities of PAO1 and HD3β without Uracil
Figure 31. Pyocyanin Production

Figure 32. Pyoverdin Production
for PAO1 with added uracil is 97%, HD3β is 6% and HD3β with added uracil is 34%.

Results for pyoverdin production are presented in Figure 32. Samples were incubated at 37°C with shaking for 12 and 30 hours. For each organism, the 30 hour points show an increase in pyoverdin production by an average factor of 4.1 over its individual 12 hour point. The points are normalized for OD$_{600}$, however there is some error introduced for the more turbid 30 hour points, because the samples were not diluted before the measurements were taken. The OD$_{600}$ values for the 30 hour points are very likely too low, which would inflate pyoverdin production values. The pyoverdin production is greatly reduced in the pyrBC' mutant without uracil added to the King’s B medium. However, with exogenous uracil supplied, pyoverdin production rises near to wild-type levels, 80% and 97% for 12 and 30 hours, respectively.

Elastase and casein protease production.

Supernatant samples for the elastase and casein protease assays were taken from cultures incubated at 37°C with shaking for 24 hours. Results for elastase and casein protease production are presented in Figures 33 and 34, respectively. The production of these two virulence factors in PAO1 both decreased when uracil was added to the medium. HD3β produced 76% the elastase of PAO1, and HD3β with uracil produced 75% the elastase of PAO1 with uracil (Fig. 33). Casein protease production was only slightly reduced for the mutant. HD3β produced 93% the Casein protease of PAO1, and HD3β with uracil produced 79% the casein protease of PAO1 with uracil (Fig. 34).

Rhamnolipid production.

Rhamnolipid production results are presented in Figure 35 and Table 3. The DHOase double knockout (DKO) strain PAO1DB92 was also used here as a comparison on the assays involving colony growth on plates (rhamnolipid production, iron chelation, blood hemolysis, swim, swarm and twitch). Because the rhamnolipid medium is a minimal medium, the
Figure 33. Elastase Production

Figure 34. Casein Protease Production
Rhamnolipid Production

Table 3. Rhamnolipid Production vs. Colony Growth

<table>
<thead>
<tr>
<th>Uracil (µg/ml)</th>
<th>Colony Average Diameter (mm)</th>
<th>Average Rhamnolipid Halo Diameter (µg/ml)</th>
<th>Halo Standard Deviation</th>
<th>Hammer Factor (HF)</th>
<th>Standard Deviation</th>
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</thead>
<tbody>
<tr>
<td>PAO1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
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<td>0.09</td>
</tr>
<tr>
<td>40</td>
<td>5.44</td>
<td>8.22</td>
<td>0.48</td>
<td>1.52</td>
<td>0.07</td>
</tr>
<tr>
<td>HD3β</td>
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<td></td>
<td></td>
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<td>5</td>
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<td></td>
</tr>
<tr>
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<td>8.58</td>
<td>1.99</td>
<td>1.98</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Figure 35 a) Rhamnolipid Production of Wild Type vs. Pyrimidine Mutants

Figure 35 b) Rhamnolipid Production of Wild Type vs. pyrB Mutant
pyrimidine mutants could not grow without added uracil. Growth was supported with both 5 and 40 µg/ml uracil, however HD3β produced no rhamnolipids (indicated by the absence of pink halo) as exhibited by PAO1. Results showed the DHOase mutant having rhamnolipid production on the same order as the wild type.

Previous studies have evaluated the ability of an organism to produce rhamnolipid qualitatively (Siegmund and Wagner 1991; Brichta 2003). In an effort to evaluate an organism’s ability to produce rhamnolipid in a quantitative manner, it was hypothesized that the magnitude of the capacity for rhamnolipid production was dependent on the amount of bacterial growth. Complicating this however, was that fact that the amount of bacterial growth was dependent on the amount of bacteria on the initial inoculation of the plate, which was impractical to equalize. Therefore, the Hammer Factor (HF) was created in an effort to normalize each rhamnolipid halo measurement by the amount of bacterial growth, much in the same way that the other exoproduct assays were normalized by OD$_{600}$ of their cultures. The HF indicates the ability of the organism to produce rhamnolipids. A higher HF means that the organism has a higher capability of producing rhamnolipids. HF is the ratio of the diameter of rhamnolipid halo to the diameter of growth. A HF of 1.0 would mean that the rhamnolipid halo is equal to the size of the growth. A HF of 2.0 would mean that the bacteria can produce rhamnolipids over an area twice the size of the area of its growth. An additional benefit to normalizing the data is that they can then be averaged. The averaged data is presented in Table 3. The results show that with normalization, the standard deviation is much lower. PAO1 and PAO1DB92 both have a HF around 1.4 and 1.5 for 5 and 40 µg/ml uracil, respectively.

Iron chelation.

CAS agar plates without uracil did not support growth for either of the mutant strains. With uracil iron chelation occurred in both strains with halos of iron clearing similar to the wild
type (Fig. 36). Previous studies have evaluated the ability of an organism to chelate iron qualitatively (Schwyn and Neilands 1987; Brichta 2003). As described above in the rhamnolipid assay, the HF was again used for the iron chelation data in an effort to evaluate each organism’s ability to chelate iron in a quantitative manner. The HF indicates the ability of the organism to chelate iron. A higher HF means that the organism has a higher capability of iron chelation. HF is the ratio of the diameter of iron clearing in the medium to the diameter of the colony. A HF of 1.0 would mean that the size of the clearing is equal to the size of the colony. A HF of 2.0 would mean that the bacteria can clear an area twice the size of the area of its growth. Based on this, the results show that HD3β has 87% the ability of PAO1 to chelate iron with 20 µg/ml exogenous uracil, and 91% with 40 µg/ml uracil (Table 4). Pyoverdin is an iron chelator, and the CAS plate results do indeed correlate with the pyoverdin results presented above and in Figure 32.

Haemolysis of blood agar.

There was no haemolysis of blood agar by HD3β, or PAO1DB92 without uracil. However, growth was supported on the tryptic soy agar (TSA) plate. PAO1 grew very well and produced haemolysin, creating a ring of lysed blood cells around the perimeter of the colony. With increasing amounts of uracil PAO1 no longer grew as well, however still haemolyzed the sheep’s blood. Both HD3β and PAO1DB92 grown on the plates with uracil exhibited haemolysis characteristics similar to the wild type as illustrated in Figure 37 and Table 5. The HF values presented in Table 5 show that the data collected at 48 hours is more reliable than those taken at 24 hours.
### Iron Chelation

**Figure 36 a) Iron Chelation of Wild Type vs. Pyrimidine Mutants**

**Figure 36 b) Iron Chelation of Wild Type vs. pyrB Mutant**

#### Table 4. Iron Chelation Capability of PAO1, HD3β, and PAO1DB92

<table>
<thead>
<tr>
<th>Nutrients</th>
<th>0 Uracil</th>
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<th>40 microg/ml Uracil</th>
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<tbody>
<tr>
<td></td>
<td>18 h</td>
<td>24 h</td>
<td>18 h</td>
</tr>
<tr>
<td>Incubation Time</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Hammer Factor (HF)</td>
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<td>Standard Deviation</td>
<td>Average</td>
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</tr>
<tr>
<td>HD3β</td>
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<tr>
<td>PAO1DB92</td>
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<td>NA</td>
<td>2.67</td>
</tr>
</tbody>
</table>

**Note:** There was no growth for the mutants without added uracil.
Haemolysis of blood agar

![Image of Haemolysis of blood agar]

Figure 37. Haemolysin Production of Wild Type vs. Pyrimidine Mutants

Table 5. Haemolysis Capability of PAO1, HD3β, and PAO1DB92

<table>
<thead>
<tr>
<th>Nutrients</th>
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<th>20 microg/ml Uracil</th>
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<td>48 h</td>
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<tr>
<td>Incubation Time</td>
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<td>PAO1</td>
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<td>HD3β</td>
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<td>PAO1DB92</td>
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Table 5. Haemolysis Capability of PAO1, HD3β, and PAO1DB92
Motility.

*Cross-streak inhibition.*

The purpose of this assay was to determine if HD3β would inhibit the growth of other organisms in the same way as the wild type. The test pseudomonad was streaked in a line, and the others to be inhibited were streaked in a line perpendicularly at a known distance from the pseudomonad. The organisms used were *B. cepacia*, *S. aureus*, and *E. coli*. The plates were incubated at 37°C for 24 hours, and the distances between the growth of the test organism and the cross-streaked organisms were measured. Results are presented in Figure 38 and Table 6. PAO1 inhibited all three organisms significantly. There was no inhibition resulting from HD3β or PAO1DB92 growth. In fact, *S. aureus* grew best in closer proximity to the pyrimidine mutants.

*Swimming.*

Results for swimming motility after 14 hours incubation at 37°C are shown in Figure 39. PAO1 has dampened swimming motility with added uracil. However, with uracil HD3β and PAO1DB92 both have increased swimming. Most notably, HD3β has improved swimming motility over the wild type without uracil. It may be that the pyrimidine mutants have increased power available for the movement of its polar (monotrichous) flagellum, or perhaps the mutants have increased expression for flagella. It would be interesting to have a look with an electron scanning microscope to view the wild type and mutants side by side. Maybe the mutants have two flagella.

*Swarming.*

Results for swarming motility after 14 hours incubation at 30°C are shown in Figure 40. PAO1 has dampened swarming motility with added uracil. With uracil HD3β and PAO1DB92 both have increased swarming. Most notably, PAO1DB92 has improved swarming motility
Cross-streak Inhibition

Figure 38. Cross-streak Inhibition of Wild Type vs. Pyrimidine Mutants

<table>
<thead>
<tr>
<th>Organism</th>
<th>B. cepacia</th>
<th>E. coli</th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inhibition (mm)</td>
<td>Distance between growth (mm)</td>
<td>Inhibition (mm)</td>
</tr>
<tr>
<td>PAO1</td>
<td>1 10 4</td>
<td>20 4</td>
<td>51 4</td>
</tr>
<tr>
<td></td>
<td>2 15 3</td>
<td>15 4</td>
<td>33 3</td>
</tr>
<tr>
<td></td>
<td>3 15 3</td>
<td>15 4</td>
<td>30 3</td>
</tr>
<tr>
<td>HD3β</td>
<td>1 0 4</td>
<td>0 3</td>
<td>0 4</td>
</tr>
<tr>
<td></td>
<td>2 0 3.5</td>
<td>0 2</td>
<td>0 3</td>
</tr>
<tr>
<td></td>
<td>3 0 3.5</td>
<td>0 2.5</td>
<td>0 3.5</td>
</tr>
<tr>
<td>PAO1DB92</td>
<td>1 0 4</td>
<td>0 4</td>
<td>0 4</td>
</tr>
<tr>
<td></td>
<td>2 0 3</td>
<td>0 3</td>
<td>0 3</td>
</tr>
<tr>
<td></td>
<td>3 0 4</td>
<td>0 3</td>
<td>0 3</td>
</tr>
</tbody>
</table>

Table 6. Cross-streak Inhibition of PAO1, HD3β, and PAO1DB92
Swimming

Figure 39. Swimming Capability

Swarming

Figure 40. Swarming Capability
matching the wild type. Swarming motility utilizes both Type IV pili and flagellum movement. Swarming motility also utilizes the exoproduct biosurficant rhamnolipid. The swarming results validated the results of the rhamnolipid assay above. PAO1DB92 showed a notable increase in rhamnolipid production with added uracil, and an affect of this is seen in the improvement in swarming motility.

_Twitching._

Results for twitching motility after 24 hours incubation at 37°C are presented in Figure 41 and Table 7. Without added uracil twitching motility of HD3β and PAO1DB92 was impaired compared to PAO1, and again improved with exogenous uracil. Twitching motility utilizes the Type IV pili also used in attachment and swarming, which would mean that HD3β should have less twitching motility than PAO1DB92 in order to be consistent with the swarming and rhamnolipid results. The results showed this to be true with HD3β having an average twitching diameter of 2.5 mm less than PAO1DB92 with and without uracil. Also of note was the large variation in phenotype observed on the twitch medium. These notes are also presented in Table 7. The phenotypes of PAO1 were opaque growth, mucoidy in appearance, yellow, and a rough growth perimeter. The phenotype for the mutants with impaired twitching motility were transparent growth, dry, “fried egg” appearance, and a smooth growth perimeter. As PAO1DB92 twitching motility was restored, so too did the phenotype return to one more similar to the PAO1 phenotype.
Figure 41. Twitching Capability

<table>
<thead>
<tr>
<th>Plate</th>
<th>PAO1 Twitch zone Dia. (mm)</th>
<th>Growth</th>
<th>HD3β Twitch zone Dia. (mm)</th>
<th>Growth</th>
<th>PAO1DB92 Twitch zone Dia. (mm)</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>19.75</td>
<td>O,M,Y,R</td>
<td>15.5</td>
<td>T,D,S,C,RL</td>
<td>17.5</td>
<td>T,M,S,C,CC</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>O,M,Y,R</td>
<td>15.5</td>
<td>T,D,S,C,RL</td>
<td>18.75</td>
<td>T,M,S,C,CC</td>
</tr>
<tr>
<td>1 + Uracil</td>
<td>21</td>
<td>O,M,Y,R</td>
<td>19</td>
<td>TO,M,R,C,L,CCRL</td>
<td>20</td>
<td>O,M,R,DC</td>
</tr>
<tr>
<td>2 + Uracil</td>
<td>21</td>
<td>O,M,Y,R</td>
<td>19.5</td>
<td>T,D,R,C,CCRL</td>
<td>20.75</td>
<td>O,M,S,DC</td>
</tr>
<tr>
<td>3 + Uracil</td>
<td>24.75</td>
<td>O,M,Y,R</td>
<td>15.25</td>
<td>T,M,R,C,CCRL</td>
<td>22.5</td>
<td>O,M,S,DC,CC</td>
</tr>
</tbody>
</table>

**Growth Codes**
- **O** opaque
- **T** transparent
- **OT** opaque and transparent
- **M** mucoid
- **D** dry
- **Y** yellow
- **C** cream
- **R** rough edges
- **S** smooth edges
- **CC** concentric circles pattern in growth
- **RL** radial lines pattern in growth
- **DC** dark cream
- **L** lobes
- **CCRL** concentric circles inside with radial lines from there to perimeter

Notes: No U - all growth about the same dia.

With U - HD3β has most outward growth

Table 7. Twitch Motility and Related Phenotypes of PAO1, HD3β, and PAO1DB92
CHAPTER IV
DISCUSSION

Creating the pyrBC' Mutant

A pyrimidine mutant of P. aeruginosa was created by first deleting large sections of sequence of pyrBC' and then by using the gentamicin cassette to achieve insertional inactivation, thereby conferring resistance to gentamicin with the incorporation of the disrupted gene. The mutated gene was cultivated in E. coli SM10 and transferred into PAO1 via biparental mating. The new mutant was identified through its auxotrophy, absolute dependence on exogenous uracil and the pyrimidine salvage pathway, and its gentamicin resistance (Gm'). The mutant was then put through two tests to verify its genetic make up. 1) A protein activity gel for aspartate transcarbamoylase (ATCase). 2) A Southern blot of the wild type and the mutant probed for pyrBC'.

Verification of the pyrBC' Mutant

Genomic PAO1 and HD3β DNA were each digested twice, once with SmaI and once with AgeI, just in case one enzyme did not work as expected. Additionally, each genomic digest was probed with 2 types of probes, A10 and HH02, just in case one probe did not work as expected. The A10 probe was created from the pA10 plasmid and contained the intact pyrBC'. The HH02 probe was created from the pHH02 plasmid and contained the mutated pyrBC', disrupted with the gentamicin cassette. Either of the probes had enough coding to bind to both the wild type and mutant forms of pyrBC'. The mutated gene probe could bind to the PAO1 digest, and the wild-type gene probe could bind to the HD3β digest. The added benefit of the mutated gene probe was the inclusion of the gentamicin cassette coding. Therefore, the mutated gene probe should also bind to any location in the HD3β genome that contained a gentamicin cassette. Remarkably, both genomic digests and both probes worked as predicted, resulting in
four independent replications of the mutated pyrB validation. Figure 19 a) and b) are pictures of the probed Southern blot. Comparing the two probed lanes of AgeI digested DH3β genomic DNA, the fact that there were no additional bands for the HH02 probe compared to the A10 probe shows that there were no gentamicin cassettes inserted in any other locations within the HD3β genome, other than in the intended pyrB. Unfortunately, the DNA ladder, New England Biolabs® (NEB) 1kb, was also hybridized by both probes. Its coding is proprietary, however, it must have a sequence homologous with a portion of pyrBC’. It was convenient to be able to see the ladder directly on the film, but a small amount of the 1kb ladder was ejected into the running buffer, and wafted into the gel at various times and locations. These are the light stair-stepping patterns seen in, and in-between lanes. The extra hybridized bands are not those of genomic DNA, but are of the 1kb ladder. The clue to this was the column of hybridized bands on the very right edge of the membrane. The right edge was not a lane, it was the portion of the gel on the outside right of the rightmost lane. Additionally, the band spacing on the edge corresponded to the 1kb ladder spacing. The stair-stepping patterns within the other lanes and lane spacers also match up to the 1kb ladder spacing. The remaining bands are hybridized genomic bands, because they were exactly the predicted sizes for the individual restriction enzymes. Additionally, the intensity of hybridized probe is significantly higher at the predicted locations.

Growth of PAO1 and HD3β

PAO1 and HD3β were grown in Pseudomonas minimal medium (Psmm) and peptone trypticase soy broth (PTSB). The mutant was able to reach wild-type optical density at 600nm (OD$_{600}$) in both media. The analysis of viable cell number (VCN) demonstrated that the mutant is also able to produce the same order of magnitude number of viable cells. Questions arise regarding the rate of growth of the cultures. Why is it, that the curves in Figure 20 have different slopes in the log phase of growth? Why does curve c) have a lower rate than b)? Perhaps a clue
is in the rate of growth of curve d). Curve d) is the same organism as c), but just with less exogenous uracil available in the medium. Perhaps c) only needs more uracil available in the medium to be able to meet the growth rate of strains shown in curves a) and b). Perhaps orotate along with uracil is required to reach the same growth rate as the wild type. Or, perhaps some other nutrient is needed. Our laboratory has preliminary results showing that PAO1DB92 grows differently when magnesium is added to the medium.

The difference in color of the mutant growth compared to the wild-type growth was the first indication that the organisms were not producing the same amounts of exoproducts. In Psmm PAO1 grows with the standard *Pseudomonas* green, a vibrant deep blue green. HD3β grows a light, mint green indicating that pyoverdin is still being produced in decreased amounts, but the pyocyanin is significantly decreased. Subsequent assays proved this to be true with 75% decrease in pyoverdin, and 94% decrease in pyocyanin (Fig. 31 and 32). HD3β grown with exogenous orotate was yet another color. It grew with the color of dark, creamy lemonade. It is hypothesized that both pyocyanin and pyoverdin are significantly reduced in this growth state. What is it that causes the exoproducts to be completely altered by a change in nutrient source? It must be because the pyrimidine pathway is regulated in a different way. This would suggest a direct link between the pyrimidine pathway and virulence. In this study, HD3β was grown in orotate, but was not assayed for pyrimidine intermediates, or exoproducts. These data would be interesting pieces to add to the puzzle.

**Analysis of the Hypothesis**

The purpose of this study was to determine the role of carbamoylaspartate (CAA) with respect to the pathogenicity of *P. aeruginosa*. The null hypothesis (H₀) was that the CAA build-up was the cause of a decreased ability of PAO1 to export virulence factors, therefore, a *pyrBC'* mutant would have the same virulence as PAO1. The alternate hypothesis (Hₐ) is that the CAA
build-up is not the cause for a decreased ability of PAO1 to export virulence factors, therefore, a
pyrBC' mutant is less virulent than PAO1. The characterization of the mutant showed that its
virulence compared to the wild type was decreased in every tested area. Thus, the H_A of this
study was accepted and the H_0 rejected. It cannot be the buildup of CAA that makes the pyrC
DKO less virulent than the wild type, because the HD3β strain is less virulent than the wild type
and has no production of CAA. Or, is the hypothesis flawed in this assumption? Perhaps it is
the buildup of CAA in the case of PAO1DB92, and something else entirely in the case of HD3β
accounting for decreased virulence. What is it then that causes these strains to be less virulent? I
believe the key is the one factor the two mutant strains have in common. That is, they are both
auxotrophic organisms. They both need to be fed exogenous uracil in order to survive. In order
to use the uracil they have to activate the salvage pathway, which has been shown to cause
regulation of the biosynthetic pathway (O'Donovan et al. 1989). The fact that the biosynthetic
pathway is down regulated makes sense. The organism needs to spend its energy creating
pyrimidines via salvage, not building up intermediates in the biosynthetic pathway. I believe that
this is also part of the reason that the mutants are less virulent. This is demonstrated by
examining the wild type. When PAO1 is fed exogenous uracil, its salvage pathway is activated,
and its biosynthetic pathway is down regulated. The result is a decrease in virulence over PAO1
in the same medium without uracil. Therefore, simply activating the salvage pathway can cause
the organism to focus more of its energy on the successful production of pyrimidines, and less on
the production of virulence factors. This is not the whole story, however, because the pyrC DKO
and HD3β strains are not less virulent than PAO1 in the same ways. They are both less virulent
than uracil fed PAO1, but PAO1DB92 is less virulent than HD3β. There must be some effect of
pyrimidine biosynthetic intermediates. The results of this study show that the pyrC DKO and
HD3β strains act very differently. When HD3β is starved for uracil all pyrimidine enzyme
specific activities are lower than wild type (Figure 30). This is in stark contrast to the increased activities seen in the DHOase mutant where all activities are increased 2 to 3 fold (Brichta 2003). The difference in virulence between HD3β and PAO1DB92 was again verified recently by an in vivo study conducted with C. elegans (Anvari 2004). In this study, C. elegans was initially fed PAO1 bacteria. There was a 100% mortality rate in the PAO1 fed nematodes. Subsequently, C. elegans was fed HD3β bacteria, resulting in 70% mortality of the nematodes. Finally, C. elegans was fed PAO1DB92 bacteria, resulting in 50% mortality of the nematodes. Clearly the virulence to nematodes was decreased for both pyrimidine mutant strains of P. aeruginosa, however PAO1DB92 yielded optimal results.

This work cannot determine conclusively what intermediate factors are potential effectors, because only enzyme activities were measured. Quantities of intermediate products were not measured (i.e. carbamoylphosphate, carbamoylaspartate, dihydroorotate, orotate). The amount of the intermediate products must be measured to determine if it is one of these which is building up and changing the energetics of the cell, as proposed by Brichta (2003), thus affecting the ability of the organism to function properly and produce virulence factors. It may indeed be the buildup of negative ions which disrupts the cell’s ability to produce exoproducts. In the pyrCDKO there is a documented buildup of CAA. Following the same logic, for a pyrB knockout there should be a buildup in carbamoylphosphate (CP), which is similarly charged (Fig. 1a). Perhaps PAO1DB92 is less virulent than HD3β, because there more negative charge produced in PAO1DB92. A key piece to the puzzle would be the measurement of carAB activity. This enzyme activity could be measured for PAO1, PAO1DB92 and HD3β to give an indication of how much difference there is in the activity of the first enzyme in the biosynthetic pathway. This would give a clue as to the overall activity of the pathway for the mutants.
The Dickstein Challenge

Dr. Rebecca Dickstein, a professor in the UNT biological sciences department, poses a valid question directed toward this research. The question is this: Is the organism less virulent, because it is an auxotroph? It is an important question and one which must be answered definitively in the continued research of the pyrimidine biosynthetic pathway and how it effects, or does not affect the virulence of an organism. In order to answer the question, another auxotroph mutant in some other pathway must be tested in the same way. If the virulence of the organism is unchanged, this would rule out auxotrophy as being the problem.

Great pains were taken in this research to rule out auxotrophy, save for examining another unrelated auxotroph. The mutant in this research is getting exogenous uracil, which is being utilized by the salvage pathway to create pyrimidines sufficient to execute all necessary cell functions to grow to the same VCN as the wild type. In the current published literature, this verification sufficiently addresses the Dickstein Challenge.

Even if auxotrophy were the reason for the noted decreased virulence, it would not make a difference to the final goals of this research. The desire is to cripple the offending organism, *P. aeruginosa*. The goal is to identify a protein in this invader’s pathway that the host does not require, and wipe out that protein, rendering the invader harmless. Whether it is because the invader has been turned into an auxotroph and is less virulent, or because it is the shutdown of the pyrimidine biosynthetic pathway that makes it less virulent, is of no immediate consequence. The results of this work can still recommend that the protein ATCase be targeted for destruction in patients infected with *P. aeruginosa*. If the organism cannot find enough exogenous uracil in the host, it will die, as demonstrated in the host *C. elegans*. If the organism can find enough uracil for flow-through to activate the salvage pathway, it will survive, however, it will not be as deleterious to the host. However, it is recommended that another auxotroph be tested in the same
ways as the pyrimidine mutants to confirm that it is not the fact that the organism needs to be fed uracil that is decreasing its virulence.

Future Work

Oligonucleotide primers were designed, which would flank the mutated pyrBC’, with the intent of using them in a polymerase chain reaction (PCR) to amplify the gene and surrounding P. aeruginosa sequence. Genomic HD3β DNA and the primers are in our laboratory ready for use. The PCR could be optimized for the genomic DNA and the amplified section of DNA sequenced.

A study of HD3β grown in exogenous orotate as a nutrient source would be beneficial. This study only went so far as to complete a growth curve for HD3β in orotate. Further investigation of orotate fed HD3β VCN, pyrimidine enzyme activity and virulence factors compared to orotate fed PAO1 could shed light on the hypothesized link between the pyrimidine pathway and virulence.

An investigation of the protein levels during the log phase of growth has been recommended by publishing scientists in the field of pyrimidine auxotrophy. Additionally, a rigorous investigation is needed of what nutrients affect the growth rate of the mutant during the log phase. It may be that a pyrimidine mutant that grows with the same log phase slope as the wild type has the same level of virulence as the wild type.

It has become clear that there are many variables responsible for the virulence factors exhibited in P. aeruginosa pyrimidine mutants. It has also become clear that these are not independent variables. It is my recommendation that a microarray chip be set up for the analysis of the pyrimidine biosynthetic pathway, salvage pathway, virulence factors, and quorum sensing genes. The changes in activities of all of these players could then be analyzed in concert for the
wild type and each individual mutant at various stages of growth. I believe this analysis would be enlightening, and necessary to find actual cause and effect relationships.

It is hypothesized by Brichta (2003) that PAO1DB92 is less virulent, because the buildup of CAA causes a buildup of negative ions, thus disrupting the cell’s energetic balance. Although there is no CAA production in an ATCase mutant, this could still be true for HD3β. The ATCase mutant should have a buildup of CP, which is also negatively charged, and could also be affecting the cell’s energetics in the same way as a CAA buildup. A good way to test this theory would be to up-regulate the arginine pathway in HD3β to see if this would alleviate the proposed CP excess and bring the virulence factor production back to wild-type levels. Alternatively, there may be no CP buildup at all. It may also be true that because the organism is fed uracil, the pyrimidine pathway is down-regulated enough, and the arginine pathway is active enough, that there is no buildup of CP. In that case the documented decreased virulence of HD3β would be for a reason other than cell energetics.

Conclusions

In conclusion, a *P. aeruginosa pyrBC'* mutant was successfully created. The mutant was characterized as an auxotroph, requiring uracil to grow. The mutant was not capable of the same growth rate as the wild type. However, given ample time, it achieved the same order magnitude of VCN as the wild type. The mutant grown in an excess of exogenous uracil was tested for each enzyme in the pyrimidine biosynthetic pathway. The results indicate that the absence of ATCase causes the elimination of dihydroorotase (DHOase) and dihydroorotate dehydrogenase (DHOdehase) activity, with an increased orotate phosphoribosyl transferase (OPRTase), orotidylate decarboxylase (OMPdecase), and ornithine transcarbamoylase (OTCase) activity. The mutant was tested for each enzyme in the pyrimidine biosynthetic pathway while starved for uracil. The results indicate that the absence of ATCase, along with no available nutrients to
salvage causes the shutdown of the pathway. Virulence of the mutant organism compared to the wild type was decreased in every tested area. Thus, the alternative hypothesis of this study was found to be true and the null hypothesis false. Further testing is required to determine whether the witnessed decreased virulence was due to auxotrophy, fewer mutant VCN than present in the wild type for the same growth period, quorum sensing, a link between the pyrimidine biosynthetic pathway and the virulence of the organism, or a combination of these factors. Although HD3β is less virulent than the wild type, PAO1DB92 is less virulent than HD3β, in both in vitro and in vivo studies. In total, the investigations of pyrimidine mutants conducted in our laboratory point to DHOase as the enzyme to target to best attack P. aeruginosa. This said, however, it is hypothesized that the most effective attack would be a combination of targets. One target in the beginning of the pyrimidine pathway to create an auxotroph, and then another in the salvage pathway to eliminate the possibility for creating pyrimidine nucleotides UTP and CTP. Perhaps upp, or ndk knocked out in combination with pyrC and pyrC2 would be the ultimate attack strategy.
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