REGULATORY DIVERGENCE OF ASPARTATE TRANSCARBAMOYLASE
FROM THE PSEUDOMONADS

DISSEITATION

Presented to the Graduate Council of the
University of North Texas in Partial
Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Andrea J. Linscott, B.S.
Denton, Texas
December, 1996
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Linscott, Andrea J., \textit{Regulatory divergence of aspartate transcarbamoylase from the pseudomonads}. Doctor of Philosophy (Biology), December 1996. 210 pp., 40 tables, 117 illustrations, references, 143 titles.

Aspartate transcarbamoylase (ATCase) was purified from 16 selected bacterial species including existing \textit{Pseudomonas} species and former species reassigned to new genera. An enormous diversity was seen among the 16 enzymes with each class of ATCase being represented. The smallest class, class C, with a catalytically active homotrimer, at 100 kDa, was found in \textit{Bacillus} and other Gram positive bacteria. In this report, the ATCases from the Gram negatives, \textit{Shewanella putrefaciens} and \textit{Stenotrophomonas maltophilia} were added to class C membership.

The enteric bacteria typify class B ATCases at 310 kDa, with a dodecameric structure composed of two catalytic trimers coupled to three regulatory dimers. A key feature of class B ATCases is the dissociability of the holoenzyme into regulatory and catalytic subunits which were enzymatically active. In this report, the ATCase from \textit{Pseudomonas indigofera} was added to class B ATCases.

The largest class, at 480 kDa, class A, contains the fluorescent \textit{Pseudomonas} including most members of the 16S rrRNA homology group I. Two polypeptides are produced from
overlapping pyrBC' genes. The former, pyrB, encodes a 34 kDa catalytic polypeptide while pyrC' encodes a 45 kDa dihydroorotase-like polypeptide. Two non active trimers are made from six 34 kDa chains which are cemented by six 45 kDa chains to form the active dodecameric structure.

Dissociation of the holoenzyme into its separate active subunits has not been possible. In this report, the ATCases from *Comamonas acidovorans* and *C. testosteroni*, were added to the class A enzymes.

An even larger class of ATCase than class A at 600 kDa was discovered in *Burkholderia cepacia*. Stoichiometric measurements predict a dodecamer of six 39 kDa polypeptides and six 60 kDa polypeptides. Unlike other large pseudomonads ATCases, the enzyme from *B. cepacia* was dissociable into smaller active forms. Both the holoenzyme and its dissociated forms were regulated by nucleotide effectors. A new class of ATCase was proposed for *B. cepacia* type enzymes.
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CHAPTER 1

INTRODUCTION

The genus *Pseudomonas* is defined as having unicellular straight, never curved Gram negative rods. With the exception of *P. mallei*, which is non-motile, all species are motile by the means of one or more polar flagella. DNA has a G + C content of 58 to 69% (78). Growth occurs from 4°C to 43°C for the genus. Pseudomonads have an oxidative, never fermentative, metabolism. Since all pseudomonads lack phosphofructokinase, they cannot utilize the Embden-Meyerhof glycolytic pathway (31). Instead, glucose is oxidized to 6-phosphogluconate and 2-keto-3-deoxy-6-phosphogluconate before forming pyruvate via the Entner-Doudoroff pathway (Figure 1). The acids derived from the Entner-Doudoroff pathway are extremely weak as compared with the mixed acid fermentation products seen in the *Enterobacteriaceae*. The combination of weak acid production and the fact that pseudomonads rapidly break down nitrogenous compounds to ammonia often mask the acid production in biochemical reactions. Hugh and Leifson found that by decreasing the peptone content of oxidative-fermentative medium and making the medium semi-solid, the small amount of acid produced was much easier to detect (53).
Figure 1. Entner-Doudoroff Oxidative Pathway

Glucose

Hexokinase

Glucose-6-phosphate

ATP

ADP

Glucose-6-phosphate

Dehydrogenase

NADP⁺

NADPH

6-phosphoglucono-δ-lactone

Lactonase

H₂O

6-Phosphogluconate

6-Phosphogluconate

Dehydrogenase

H₂O

2-Keto-3-deoxy-6-phosphogluconate (KDPG)

KDPG

Aldolase

Glyceraldehyde-3-phosphate

Pyruvate
Molecular oxygen serves as the terminal electron acceptor in all species; however, some species can use nitrate under anaerobic conditions as an alternative final electron acceptor.

Prior to an intensive study (120), the genus *Pseudomonas* contained over 200 species which included Gram positive organisms, fermentative organisms, and peritrichous organisms (11). The work of Stanier, Palleroni, and Doudoroff attempted to revamp the hodgepodge genus. Their work built on the nutritional versatility seen in these organisms as previously noted by den Dooren de Jong (24). They also incorporated other physiological and biochemical tests as taxonomic tools. These tests included: pigmentation, test for denitrification, ortho- and meta- cleavage of aromatic compounds used to screen for ring fission mechanism, the presence of arginine dihydrolase, oxidase test, the production of extracellular enzymes, determination of base composition of DNA, and the ability to accumulate poly-ß-hydroxybutric acid as cellular reserve.

Ribosomal RNA-DNA hybridization was then used to group species of the *Pseudomonas* genus into five RNA homology groups (Table 1). Of the 32 organisms listed in the RNA homology groups, I have chosen 16 pseudomonads and former pseudomonads for my work. These are denoted by asterisks in Table 1.

*Pseudomonas aeruginosa*

In 1872, Schroeter reported a water-soluble, blue-green pigment on a sliced boiled potato and in pus. Although he never isolated or observed the organism under a microscope, he
Table 1. Original RNA Homology Groups (97)

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<tr>
<td><strong>Fluorescent pigmentation group</strong></td>
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<tr>
<td><em>P. aeruginosa</em></td>
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<td><em>P. putida</em></td>
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<td><em>P. fluorescens</em></td>
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<td><em>P. chlororaphis</em></td>
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<td><em>P. syringae</em></td>
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<td><em>P. cichorii</em></td>
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<td><em>P. stutzeri</em></td>
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<td><em>P. mendocina</em></td>
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<td><em>P. alcaligenes</em></td>
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<td><em>P. pseudoalcaligenes</em></td>
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<tr>
<th>RNA Homology group II</th>
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<td><em>P. pseudomallei</em></td>
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<td><em>P. caryophylli</em></td>
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<td><em>P. gladioli</em></td>
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<td><em>P. pickettii</em></td>
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<td><em>P. solanacearum</em></td>
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<td><em>P. facilis</em></td>
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<td><em>P. pseudoflava</em></td>
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<td><em>P. diminuta</em></td>
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<th>RNA Homology group V</th>
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<td><em>P. maltophilia</em></td>
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<th>RNA Homology - never grouped, 65 species listed</th>
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<td><em>P. indigofera</em></td>
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<td><em>P. putrefaciens</em></td>
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never isolated or observed the organism under a microscope, he
called this pigment producing "organism" Bacterium aeruginosum
(109). In 1882 a blue-green exudate from a surgical wound
contained an organism which Gessard named Bacillus pyocyaneus
(40). Migula published the combined name as Pseudomonas
aeruginosa (84) and thereby established that the neotype must
produce pyocyanin and that the organism be a motile rod.
Subsequent work required that the organism must be a Gram
negative rod and be motile by means of polar flagella.

Often pigment production is a key identification
characteristic for P. aeruginosa. Pyocyanin is a water soluble
blue-green pigment unique to P. aeruginosa. Other pigments may
be produced, including, pyoverdin (yellow), pyorubrin (red),
and pyomelanin (brown). However, there are some strains that
do not produce any pigment. Pigments are thought to function
as iron scavengers and as virulence factors. Strains of P.
aeruginosa typically produce a distinct grape-like odor which
is associated with the production of 2-aminoacetophenone. On
agar plates, P. aeruginosa often exhibit a metallic sheen, due
to autolysis of the organism. Growth at 42°C is a useful
identification characteristic, while a flagellar stain reveals
one polar flagellum for this species. All P. aeruginosa
strains are oxidase positive. Other differential
characteristics or distinct biochemical tests include: positive
arginine dihydrolase, positive denitrification, positive
gelatin hydrolysis, polymyxin susceptible, no β-hydroxybutyrate
accumulation, ortho cleavage for both catechol and procatechuate, and G + C content of 67.2%.

*P. aeruginosa* may infect a variety of host cells including plants, insects, fish, amphibians, reptiles, birds, mammals, and is considered an opportunistic pathogen in humans. Thus, *P. aeruginosa* lacks the ability to initiate an infection in healthy immunocompetent people. However, in persons having cystic fibrosis, a genetic disorder in which there is a defect in the transmembrane regulator protein that results in thick mucous secretions (23), *P. aeruginosa* is able to colonize the patient's lung because thick mucus has impaired the ciliated cell clearance mechanism. Once *P. aeruginosa* has colonized the lungs, the organism is protected from the host defenses such as phagocytosis either by the thick mucus itself or by some strains producing an alginate - slime layer. In persons with serious burns, where the normal epithelial skin barrier has been destroyed, *P. aeruginosa* is a frequent cause of infection. It is common for persons under immunosuppressive therapy and cancer treatment, to acquire a heavy nasopharyngeal colonization of *P. aeruginosa* which can lead to a systemic infection. Once *P. aeruginosa* enters in the bloodstream death usually occurs due to septic shock. The type species, *P. aeruginosa*, is also a causative agent of infection whenever indwelling devices are used (41). For example, *P. aeruginosa* can cause urinary tract infections when catheter devices are used or can cause pneumonia when respiratory devices are used (41). There are many
virulence factors that are involved with \textit{P. aeruginosa} pathogenicity (41). Exotoxin A inhibits protein synthesis which leads to tissue destruction and aggravates the host’s inflammatory response. Proteases and elastases, produced by \textit{P. aeruginosa}, destroy tissue, while the pigments produced often inhibit the growth of other bacteria.

\textit{Pseudomonas putida}

Only a negative gelatinase reaction separated \textit{Bacillus fluorescens non-liquefaciens} from \textit{B. fluorescens liquefaciens} (35). Trevisan also isolated the same strain which he called \textit{Bacillus putidus} (124). The current name was designated in 1895 as \textit{Pseudomonas putida}, which was later divided into two biotypes. In 1900 and 1901, there was much activity with this particular strain. Each person who isolated the strain gave it a new name. For example, \textit{P. putida} which has a distinct oval appearance on Gram stain, was called \textit{Pseudomonas ovalis} (20). All strains were later shown to be synonymous with \textit{P. putida}.

The organism is part of the fluorescent pseudomonads in the rRNA homology group I. The yellow–green fluorescent pigment produced by \textit{P. putida} functions as a siderophore, which binds and transports ferric iron. As Flugge noted, \textit{P. putida} is so similar to \textit{P. fluorescens} that a negative gelatinase reaction and no growth on either trehalose and meso-inositol separate \textit{P. putida} from the latter. Other differential characteristics or distinct biochemical tests for \textit{P. putida} include: positive oxidase, growth at 4°C, positive arginine dihydrolase, polymyxin
susceptible, no poly β-hydroxybutyrate accumulation, **ortho**
cleavage of both catechol and procatechuate, and G + C content
of 62.5%.

The *P. putida* species is usually isolated from
environmental sources such as soil and water and is rarely seen
as an opportunist pathogen. Recently however, the organism
has been recognized as an opportunist pathogen in cancer
patients (2) and has been reported to caused septicemia following
a blood transfusion (41). Pathogenicity is partly due to an
adhesive exopolysaccharide which allows the organism to adhere
to mucosal membranes and promotes colonization of the organism.
Another virulence factor that causes pathogenicity in *P. putida*
is lipopolysaccharide (LPS) endotoxin (41). LPS endotoxin, when
released into the bloodstream, can cause septic shock that can
lead to death.

*Pseudomonas fluorescens*

As mentioned above, Flugge discovered two bacterial
strains so similar that the only test to differentiate them was
gelatin hydrolysis. He named the strain that could hydrolyze
gelatin *Bacillus fluorescens liquefaciens* and the strain that
could not hydrolyze gelatin *Bacillus fluorescens non-
liquefaciens* (35). The gelatin hydrolyzing organism was also
isolated by Trevisan (124), who named the organism *Bacillus
fluorescens*. The name of the organism was later changed to
*Pseudomonas fluorescens* (83) which has been further divided
into seven biovars (A through G).
A water-soluble fluorescent pigment is produced by *P. fluorescens* when it is exposed to UV radiation at a wavelength of 254 nm. This fluorescent pigment may be yellow-green, yellow-brown, or be nearly colorless. Often pigment production is influenced by the nutrients available or medium composition. The organism is oxidase positive with one to six polar flagella and is usually resistant to carbenicillin but susceptible to kanamycin. The optimal temperature for growth is 30°C with no growth at 42°C, though some strains can grow at 37°C. Other differential characteristics or distinct biochemical tests include: positive arginine dihyrolase, positive lysine decarboxylase, denitrification, gelatin hydolysis, positive lecithinase, polymyxin susceptible, *ortho* cleavage of both catechol and procatechuate, and G + C content of 59.4 - 61.3%.

The organism is usually found in the soil and in water and has been implicated in the food spoilage of fish and meat. It has also been isolated clinically, usually from invasive procedures (catheters and respirators) or from post-operative infections. Proteolytic enzymes are released as virulence factors to destroy tissue (41).

*Pseudomonas aureofaciens*

While working in Kluyver's laboratory, Bouman incidentally isolated a new pseudomonad (64). He was experimenting with a mixture of clay and heat-sterilized kerosene, which he incubated at 25°C for 3 weeks (64). After which, the kerosene was poured off and the clay resuspended
in sterile water. This clay slurry was streaked onto a peptone agar plate containing glucose and then incubated at 30°C. After two days, colonies began to appear which produced a brownish-yellow pigment. The pigment isolated from the liquid medium was later identified as phenazine-α-carboxylic acid. Because it was war time when this work was done, these data were never reported. It was not until 1956 that there was renewed interest in this organism. Haynes and coworkers reported the isolation of an organism that produced a golden pigment, phenazine-α-carboxylic acid (49). A collaborative effort was then begun with Kluyver, which resulted in the finding that both organisms were the same. Based on Gram stain morphology and the pigment produced, _P. aureofaciens_ was the name proposed (64). The aerobic taxonomic study of Stanier et al. (120) treated _P. aureofaciens_ as _P. fluorescens_ biotype E since both organisms were very similar. However, the current Bergey's Manual of Systematic Bacteriology, Volume I (93) treats _P. aureofaciens_ as a separate species, as I do in this research.

The organism can be found in the soil and there has been no reported cases to date of human infection caused by _P. aureofaciens_. Differential characteristics or distinct biochemical tests include: positive oxidase, growth at 42°C, arginine dihydrolase, lysine decarboxylase, gelatin hydrolysis, accumulation of poly-β-hydroxybutyrate, polymyxin susceptible,
ortho cleavage of both catechol and procatechuate, and G + C content of 63.6%.

Pseudomonas syringae

Early plant pathologists assumed that interactions between plants and microorganisms were highly specific and that an organism could infect only a certain host plant. Thus, many of the same bacterial strains were given different names depending on what type of host plant was infected. Such is the case for P. syringae, which has an impressive list of over 40 synonyms in the 8th edition of Bergey's Manual of Determinative Bacteriology (27). Bergey's Manual of Systematic Bacteriology, Volume I lists these synonyms as pathovars for P. syringae (93). The organism was first isolated from lilacs (126) and can infect a variety of plants including: peas, beans, soy beans, blackberry, tomato, sunflower, tobacco, coffee bean plants, and wheat (27). The phytopathogenic strains produce lesions that later become necrotic due to the release of toxic metabolites from the organism (48). A hypersensitivity reaction, which amplifies the tissue damage, is always seen in tobacco plants infected with P. syringae (27).

The organism is a member of the fluorescent pseudomonads in the rRNA homology group I. Its optimal temperature is 25 to 30°C. A slime layer is produced when the organism is grown in 2 to 4 % sucrose. A negative arginine dihydrolase reaction and a negative oxidase reaction set this species apart from the other fluorescent pseudomonads. Other differential
characteristics or distinct biochemical tests include: one to six flagella, ortho cleavage of procatechuate, and G + C content of 59 - 61%.

*Pseudomonas stutzeri* and *Pseudomonas mendocina*

The 'stutzeri group' is composed of two species: *Pseudomonas stutzeri* and *P. mendocina*. The former, *P. stutzeri*, was previously named *Bacillus dentrificans* II (14) and *Bacterium stutzeri* (70) before its current designation to the genus *Pseudomonas* (127). The latter, *P. mendocina*, was isolated by Palleroni and Solanes from soil and water in the province of Mendoza, Argentina (95). Both strains are non-fluorescing and are capable of growing under strict anaerobic conditions in nitrate-containing media. The 'stutzeri group' is part of the rRNA homology group I.

*Pseudomonas stutzeri* possesses a polar or lateral flagellum depending on the type of growth medium used. On solid medium, a lateral flagellum is observed (116). *P. stutzeri* appears as dry, wrinkled, and adherent colonies on agar plates although a smooth variant of the organism occasionally appears. Due to the high cellular concentration of cytochrome c, the colonies typically have a buff to light brown color on agar medium. Other differential characteristics or distinct biochemical tests include: oxidase positive, growth at 4°C, denitrification, growth in 6.5% NaCl, no accumulation of poly-β-hydroxybutyrate, polymyxin susceptible,
ortho cleavage of both catechol and procatechuate, and G + C content of 60.6 - 66.3%.

The organism has been isolated from water, soil, animals, hospital equipment, and human clinical specimens. Most human clinical infections are of an opportunistic nature which include: septicemia caused by contaminated intravenous fluid, lesions in diabetic patients, urinary tract infections associated with catheters, endocarditis caused by contaminated prosthetic valve, and puncture wound osteomyelitis (41).

Strains of *P. mendocina* produce flat, smooth, and non-wrinkled colonies on agar medium. They produce a yellowish-brown pigment due to intracellular carotenoid pigment. A polar or lateral flagellum is produced, depending on growth conditions. Because of their similarities, *P. mendocina* has sometimes been confused with denitrifying biovars of *P. fluorescens*. Growth at 42°C and possession of a polar monotrichous flagellum help distinguish the two. Other differential characteristics or distinct biochemical tests include: oxidase positive, growth at 4°C, arginine dihydrolase positive, ornithine decarboxylase positive, growth in 6.5% NaCl, no accumulation of poly-ß-hydroxybutyrate, polymyxin susceptible, ortho cleavage of both catechol and procatechuate, and G + C content of 62.8 - 64.3%.

*P. mendocina* is rarely recovered as a human pathogen, but is sometimes recovered from environmental sample as the
organism is found in water and soil (41). The organism has also been isolated from diseased fleece of sheep

*Pseudomonas pseudoalcaligenes*

*Pseudomonas pseudalcaligenes* is part of the 'alcaligenes group' which also contains the species, *P. alcaligenes*. Both *P. pseudoalcaligenes* and *P. alcaligenes* are closely related to the 'stutzeri group' (103) and are part of the rRNA homology group I.

Strains of the alcaligenes group are characterized by their ability to grow at 41°C, inability to utilize carbohydrates, and by their limited nutritional spectrum. Although *P. pseudoalcaligenes* closely resembles *P. alcaligenes*, it can be differentiated from *P. alcaligenes* by its ability to produce acid in fructose oxidative-fermentative (OF) medium, arginine dihydrolase, a lower G+C content of 62 - 64%, and a slightly broader nutritional spectrum. Other differential characteristics or distinct biochemical tests include: oxidase positive and polymyxin susceptible.

The organism has been isolated from food, water, animals, hospital equipment, and human clinical samples. The type species, *P. pseudoalcaligenes*, was isolated from a patient with sinus drainage. *P. pseudoalcaligenes* has also been implicated as the causative agent of meningitis, septicemia, pneumonitis; it was also isolated from a post-operative knee infection (41). Curiously, there is also a
strain of *P. pseudoalcaligenes* that is pathogenic for watermelon (*Citrullus lanatus*) (27).

**Burkholderia cepacia**

In 1950 Burkholder reported that *Pseudomonas cepacia* was the causative agent of onion rot (13) while a new species, *Pseudomonas multivorans* was described for the first time (120). Except for the ability to cause root rot in onion bulbs, there were no differences between the two species. Subsequent experiments showed that *P. multivorans* could indeed cause onion rot. *Pseudomonas multivorans* (120) and *Pseudomonas kingii* (58) were later shown to be synonymous with *P. cepacia* (92, 114).

*Pseudomonas cepacia* is one of the most nutritionally versatile pseudomonads. The organism is capable of utilizing over 150 organic compounds for carbon and energy (4). This nutritional versatility is likely to be due to the organism's fluid genome (104). Although no fluorescent pigment has been observed, various yellow, brown, red, and purple pigments have been seen. Unlike most pseudomonads which are oxidase positive, *P. cepacia* is oxidase variable. Several cell wall chemical differences can be seen in *P. cepacia* as compared to the other pseudomonads. For instance, the major components of the cell wall of *P. cepacia* are myristic, 3-hydroxymyristic, and 3-hydroxypalmitic acid which are not seen with other pseudomonads. Likewise, hopane, a triterpene derivative similar to sterols in size and function, is found only in the cell wall of *P. cepacia*. The organism grows slowly on agar medium and is often non-
viable after 3-4 days on solid media. Other differential characteristics or distinct biochemical tests include: one to seven polar flagella, positive for lysine and ornithine decarboxylase, gelatin hydrolysis, accumulation of poly-β-hydroxybutyrate, polymyxin resistant, ortho cleavage of protocatechuate, and G + C content of 67.4%. Based on DNA and rRNA homology studies, *P. cepacia* was placed in RNA homology group II. The entire RNA homology group II has now been placed in a new genus, *Burkholderia*. Thus *P. cepacia* has now become *Burkholderia cepacia* (141).

*B. cepacia* has a wide geographic distribution. It was been isolated from soil, pasteurized milk, contact saline solutions used to wash contact lens, rotting tree trunks, rivers, and tap water. Nosocomial infections such as septicemia, meningitis, endocarditis, pneumonia, and urinary tract infections are caused in part by *B. cepacia*’s nutritional diversity. The species can be found growing in distilled water, disinfectants, aerosol polymyxin, anesthetics, detergent solutions, respirators, humidifiers, urinary catheter kits, baby lotion, and flower vases. The organism has emerged as an important pulmonary pathogen in persons with cystic fibrosis (CF) (41, 43, 56, 123). Isolation of *B. cepacia* from patients having CF is associated with the rapid deterioration in pulmonary status and death (45). A study in 1982 showed that up to 27% of all CF patients were infected with *B. cepacia* (5). This percentage
is misleading because at that particular time, *B. cepacia* was hard to isolate, in that it is a slow growing organism that is often overgrown by other pseudomonads (i.e. *P. aeruginosa* and mucoid *P. aeruginosa*) commonly isolated from respiratory samples of CF patients. Since most pseudomonads are susceptible to polymyxin B and *B. cepacia* is resistant, polymyxin B supplemented with other inhibitory substances are added to an agar medium in order to recover the organism from clinical specimens. It was not until a medium specific for the isolation of *B. cepacia*, called PC medium, became available that the recovery of *B. cepacia* was enhanced (44). For *B. cepacia* the exact mechanism for pathogenicity is unknown. Proteolytic and lipolytic activities are observed, but unlike *P. aeruginosa*, neither elastase nor toxin A is produced. What makes this organism so serious for CF patients is its resistance to both conventional and experimental antimicrobial therapy (41, 43, 45).

An unprecedented feature of *B. cepacia* is its genome mobility (104). The genomes of *B. cepacia* strain ATCC 25416 and strain ATCC 17616 contain a large number of insertion sequences (IS) which afford the organisms with the ability to mobilize and in recruit foreign genes (19, 73). Moreover, these IS are involved in the rearrangement of the genome and replicon fusion (19, 73). The acquisition of new genetic material not only enhances nutritional diversity, but also contributes to the organism's ability to become resistant to
antimicrobials. *B. cepacia* has an 8.1 megabase (Mb) genome, twice the size of the genome of the average bacterium (21, 104). Strain 17616 of *B. cepacia*, has a slightly smaller genome of 7 Mb as compared to *B. cepacia* 25416 (19). Although different sizes, both *B. cepacia* strains have three large circular chromosomes and one large cryptic plasmid.

Because of its unusual genetic characteristics, the organism has the capacity to use many different substrates for carbon and energy sources. For this reason, *B. cepacia* has become an excellent candidate for bioremediation. However, these same attributes which can be exploited in industry for bioremediation, can have disastrous consequence in contributing to human disease. Once the organism establishes itself in the human body, it can withstand most antimicrobial treatments and continue to thrive.

Only a few genes have been isolated and characterized from the organism. This makes *B. cepacia* a fertile ground for new discoveries, if one has the patience to work with an organism with three genomes!

*Burkholderia pickettii*

In the 1970's a new pseudomonad strain was isolated from clinical sources. *Pseudomonas thomasii* was the tentative name of the organism isolated from autoclave-cooling water (100). Pickett collected 35 unidentified denitrifying organisms from various clinical sources including, tracheotomy wounds, blood, and hospital equipment. He identified these organisms as *P.*
*Pseudoalcaligenes* type 2. Pickett's organisms were then determined to be related to *P. solanacearum*, an RNA homology group II organism. The organism was then given the name *P. pickettii* and eventually placed in RNA homology group II (102). A later study showed that *P. thomasii* was the same organism as *P. pickettii* (62). In 1992 the entire RNA homology group II was moved to a new genus, *Burkholderia* (141). Thus, the name has now become *Burkholderia pickettii* which can be further divided into seven biovars. The biovars are separated on the basis of their oxidative patterns.

*Burkholderia pickettii* shares the vast nutritional versatility with other members of the RNA homology group II. The bacterium grows slowly on agar medium and becomes non-viable after 3 - 4 days on plates. Because the organism is a slow denitrifier and produces delayed carbohydrate oxidation reactions, *B. pickettii* was often misidentified as the non-reactive *P. pseudoalcaligenes*. Two polar flagella are usually observed with this strain. Other differential characteristics or distinct biochemical tests include: oxidase positive, growth at 42°C, accumulation of poly-β-hydroxybutyrate, polymyxin resistant, ortho cleavage of protocatechuate, and G + C content of 64.

The organism is usually isolated from clinical sources and has been the cause of septicemia, meningitis, urinary tract infections, and respiratory infections. The organism has been isolated from various types of hospital equipment - autoclave,
cooling machines, artificial kidney machines, intravenous fluids, and dialysis fluid (41).

*Comamonas acidovorans* and *Comamonas testosteroni*

The *Pseudomonas acidovorans* group consist of two species: *P. acidovorans* (24) and *P. testosteroni* (79). The acidovorans group became part of the rRNA homology group III which contains the hydrogen utilizing pseudomonads and the non-hydrogen utilizing pseudomonads - the acidovorans group (93). De Vos, et al., showed that both *P. acidovorans* and *P. testosteroni* shared the same 16S rRNA subdivision and fatty acid composition as did *Comamonas terrigena* (26). De Vos proposed reinstatement of the genus *Comamonas*, with *C. terrigena* as the type species. Later studies compared *P. acidovorans*, *P. testosteroni*, and *C. terrigena* with regard to: DNA-DNA hybridization, cellular fatty acid composition, quinone composition, and the electrophoretic mobility patterns of certain enzymes (122). Based on the data obtained from that study, both *P. acidovorans* and *P. testosteroni* were moved to the genus *Comamonas*. In 1991 a new family, *Comamonadaceae*, was created for these organisms (138).

Both *C. acidovorans* and *C. testosteroni* are non-pigmented, have lophotrichous flagella, and are nutritionally versatile. Although both strains show nutritional diversity, neither strain can degrade glucose or other carbohydrates as other pseudomonads do. *Comamonas testosteroni* is unique in being able to grow on testosterone and other steroids. *Comamonas acidovorans* can be differentiated by the organism's ability to assimilate D-
fructose, D-mannitol, DL-tartrate, ethylene glycol, and propylene glycol. *C. terrigena* requires methionine and nicotinamide for growth. Other biochemical tests or characteristics for *C. acidovorans* include: positive oxidase, one to six flagella, accumulation of poly-β-hydroxybutyrate, meta cleavage of protocatechuate, and G + C content of 67. Other biochemical tests or characteristics for *C. testosteroni* include: oxidase positive, one to six flagella, no accumulation of poly-β-hydroxybutyrate, polymyxin susceptible, meta cleavage of protocatechuate, and G + C content of 62.

Both organisms can be isolated from soil and water. Although *C. acidovorans* and *C. testosteroni* have been isolated from hospital equipment - intravenous tubing and urinary catheters, rarely have these two organisms been implicated as the causative agent for an infection. There has only been one reported case of septicemia caused by *C. acidovorans* and one reported case of conjunctivitis caused by *C. testosteroni* (41).

*Brevundimonas diminuta*

In 1954 Leifson and Hugh noted organisms cultured from fresh-water streams that had an unusual polar flagellum (71). This organism had a tightly-coiled flagella that originated more off-centered than in the center of the pole. The measurement of the curvature of the flagellum was shown to be 0.62 mm as opposed to 2 mm which is typically seen with polar monotrichous flagella. This gave the flagellum a more tightly-coiled appearance (71). Biochemical tests revealed that the
organism could liquefy gelatin and oxidize ethanol to acids. Based on those observations, Leifson and Hugh suggested that the organism be placed in the genus *Pseudomonas* with the species being *diminuta*. The diminuta group included two species: *Pseudomonas diminuta* and *P. vesicularis* (4), which were placed in the RNA homology group IV (97). The diminuta group had distinct characteristics that have not previously been seen in the pseudomonads. The tightly-coiled monotrichous flagellum was the group's key feature. The organism also had growth requirements for pantothenate, cystine, biotin, and cyanocobalamin. Unlike the other pseudomonads, the diminuta group had a limited nutritional spectrum. *P. diminuta* could be distinguished from *P. vesicularis* by its requirement for cystine and a lack of a carotenoid pigment. *P. diminuta* accumulates poly-β-hydroxybutyrate and has a G + C content of 66.3 - 67.3.

Even in 1968, it was thought that the genus *Pseudomonas* was too broad, but researchers felt that there was still insufficient information on which new genera should be created (4). In 1994, the diminuta group had been moved to the newly created genus *Brevundimonas* (112). *Brevundimonas* refers to a bacterium with a tightly-coiled flagellum. The genus differs from the authentic pseudomonads by having a unique polar flagellum, limited biochemical activity, and different fatty acid composition.
**Pseudomonas indigofera**

Aptly named because of its deep blue pigment, *Bacillus indigoferus* was first isolated in 1895 (130). A more detailed description of the organism followed along with a name change to *Pseudomonas indigofera* (28). The genus *Pseudomonas* is tentative as no rRNA homology group could be found (93). The organism produces a blue pigment when grown at 18 - 20°C and no pigment when grown at 37°C (93). The species produces blue metallic colonies on agar plates, which remain imprinted in the medium after the colony has been scrapped off. Most of the literature on this species discusses pigment production only but there are some other characteristics are listed. These attest that the organism has one polar flagellum, is oxidase positive, and is capable of using nitrate under anaerobic conditions. No other biochemical tests used for identification have been reported.

**Shewanella putrefaciens**

First reported as the causative agent for rancid butter (25), *Pseudomonas putrefaciens* was later shown to be the primary cause of spoilage of foods stored at cool temperatures (2 - 4°C) such as butter, fish, meat, and poultry (114). Although the organism was placed in the genus *Pseudomonas*, an rRNA homology group was not assigned. Later, the organism was placed in the genus *Altermonas*, a genus similar to *Pseudomonas*; however, there is a huge difference in DNA base composition. *Altermonas* has a lower G + C content of 43 - 48 as opposed to G + C content of > 60 typically seen in the pseudomonads (68). A new genus,
Shewanella was proposed (77) and was supported by additional analysis of polar lipids, fatty acids, and isoprenoid quinones which clearly showed a difference between *S. putrefaciens* and other *Altermonas* species (88). Thus, the organism is now referred to as *Shewanella putrefaciens*.

This organism is unique in being the only non-fermentative organism capable of producing hydrogen sulfide on triple sugar iron agar (TSI), which could lead to an erroneous identification as a member of the *Enterobacteriaceae* if the oxidase test were not performed. The oxidase reaction for *S. putrefaciens* is always positive. The organism appears as a red-brown or pink mucoid colonies on agar medium and has been divided into two biovars, which differ on the oxidation of sucrose and maltose and the ability to grow at high salt (6.5 %) concentrations. Other differential characteristics or distinct biochemical tests include, growth at 4°C and 42°C, ornithine decarboxylase, and polymyxin susceptible.

The organism is primarily isolated from dairy and fishery sources and has been implicated in a few human clinical infections. It has caused meningitis in a person with severe head injuries, intra-abdominal abscess in a patient with biliary tract disease, and septicemia in a patient with chronic leg ulcers (41). In each case the patient had an underlying problem, which allowed the organism to invade tissues and cause disease.
"Stenotrophomonas maltophilia"

"Bacterium bookerii", an organism isolated from pleural fluid by J. L. Edwards in 1943, was later classified as *Pseudomonas maltophilia* (54). *P. maltophilia* was placed in the rRNA homology group V. There was concern at the time, that *P. maltophilia* was more closely related to the genus *Xanthomonas*, which is a well defined group of yellow pigmented phytopathogenic pseudomonads. Eventually, *P. maltophilia* was transferred to the genus *Xanthomonas* due to similarities in rRNA sequence patterns, sensitivity to phage typing, and the composition of the cell envelope (121). The definition of the genus *Xanthomonas* was broadened to allow *X. maltophilia* a place in the genus. Therefore, a new genus was created to place *X. maltophilia* and to allow the definition of the genus *Xanthomonas* to return to its original scope. *Stenotrophomonas*, which means unit feeding on few substrates, was the new proposed genus (94). The name was given in response to *S. maltophilia*'s limited nutritional spectrum as seen in the aerobic taxonomic study by Stanier, et al. (120). Not only does *S. maltophilia* have a limited nutritional spectrum, but most strains require methionine for growth; there have been noted exceptions some strains do not require methionine (55).

On nutrient agar plates, *S. maltophilia* has a yellow pigment. When the organism is grown on blood agar, a strong ammonia odor is produced. Other differential characteristics
or distinct biochemical tests include: oxidase negative, one to six flagella, lysine decarboxylase positive, hydrolysis of esculin and DNA, polymyxin susceptible, and G + C content of 67.

*Stenotrophomonas maltophilia* has been isolated from water, soil, animals, plants, pharmaceuticals, hospital equipment, and human clinical specimens. After *P. aeruginosa*, *S. maltophilia* is the most frequently isolated pseudomonad from human clinical specimens (52). *S. maltophilia* has been the causative agent for primary pneumonia and numerous nosocomial infections. Nosocomial infections included, endocarditis, aspiration pneumonia, urinary tract infections, wound infections, and sepsis (41). Pathogenicity of the organism can be attributed to the following enzymes: elastase, esterase, lipase, hyaluronidase, and RNAse.

**Taxonomic Studies**

Previous taxonomic studies have relied on biochemical properties, ribosomal RNA, and phenotypic characteristics to show relatedness among various *Pseudomonas* species (120). Numerical taxonomy has also been used with some success for showing relatedness among the various *Pseudomonas* species (69, 76, 86). Such taxonomic studies involve the collection of many morphological, biochemical, physiological, serological, and other related data for different bacterial strains. Each datum point is analyzed on an equal weight basis to determine
similarity values for each bacterial strain. These similarity values are then used to group bacteria by overall relatedness. Metabolic pathways and their regulatory properties can also be used as taxonomic probes. When an essential enzyme is found in all organisms, it can be assumed to have originated from a common ancestor and that its regulation could serve as a taxonomic tool (16, 74). For example, the β-ketoadipate pathway, which is used to dissimilate aromatic compounds, was one of the first biochemical pathways to be used to determine relatedness among pseudomonads (16). Since that time, tyrosine biosynthesis (15), phenylalanine biosynthesis (134), and 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase (DAHP) (133) have also been used to show relatedness among pseudomonads. Just as aspartate transcarbamoylase (ATCase) has been shown to be useful in determining relatedness among members of the Enterobacteriaceae (32, 34, 135) and Yersinia enterocolitica and Y. enterocolitica-like organisms (33), I show that the properties of ATCase are appropriate to demonstrate relatedness among species of Pseudomonas and other members of the γ-3 subgroup of the Proteobacteria (139). This is important because the Pseudomonas genus is in a state of flux at present. The entire rRNA homology group II has been placed in a new genus, Burkholderia (135), the former pseudomonads of rRNA homology group III have either been placed in the genus Comamonas (26, 138) or the genus Hydrogenophaga (137). The former rRNA homology group IV has been placed in
the new genus, *Brevundimonas* (112). Some former pseudomonads have had more than one move to new genera, the former *Pseudomonas maltophilia* was once placed in the genus *Xanthomonas* (121) only to be placed in a new genus *Stenotrophomonas* (94) later. *Pseudomonas putrefaciens*, which never had an assigned rRNA homology group, was first placed in the genus *Altermonas* (68) and then in the *Shewanella* genus (77). Soon, only a few fluorescent pseudomonads and a few non-fluorescent pseudomonads will be regarded as bona fide members of the *Pseudomonas* genus.

Aspartate Transcarbamoylase

Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the first reaction unique to pyrimidine biosynthesis (Figure 2), namely the condensation of aspartate and carbamoylphosphate to yield carbamoylaspartate and inorganic phosphate. Aspartate transcarbamoylase exists as either a single monofunctional enzyme in bacteria or as part of a multienzyme complex in higher organisms. In mammals, the third biosynthetic enzyme dihydroorotase (DHOase) is fused to a carbamoylphosphate synthetase (CPSase)-aspartate transcarbamoylase (ATCase) protein to form a multienzyme complex referred to as CAD (22, 87, 108). In higher organisms the enzymes for pyrimidine biosynthesis de novo tend to be multifunctional proteins that are thought to have arisen by through gene fusion (57). In some higher organisms such as *Saccharomyces cerevisiae*, the
FIGURE 2. Pyrimidine Biosynthetic Pathway

Dihydroorotate dehydrogenase

\( pyrD \)

\[ \text{NADH} + \text{H}^+ \]

\[ \text{NAD}^+ \]

Orotate

\( pyrE \)

\[ \text{PRPP} \]

Orotate phosphoribosyltransferase

\[ \text{PPi} \]

Dihydroorotate

\( pyrC \)

\[ \text{H}_2\text{O} \]

Dihydroorotase

Carbamoyl aspartate

\( pyrB \)

\[ \text{Pi} \]

Aspartate transcarbamoylase

Carbamoylphosphate

\( pyrA \)

\[ \text{2 ATP, HCO}_3^- \text{,} \]

\[ \text{Gln or NH}_3 \]

Carbamoylphosphate synthetase

\( pyrG \)

\[ \text{CTP} \]

Nucleoside diphosphate kinase

\[ \text{ADP} \]

CTP synthetase

\[ \text{ADP} \]

\[ \text{Pi} \]

UDP

\( ndk \)

\[ \text{ATP} \]

\[ \text{ADP} \]

\[ \text{CTP} \]

\[ \text{AMP} \]

\[ \text{CO}_2 \]

\[ \text{UMP} \]

\[ \text{UMP Kinase} \]

\[ \text{ADP} \]

\[ \text{UTP} \]

\[ \text{ATP} \]

\[ \text{NH}_3 \]

\[ \text{ADP} \]

\[ \text{Pi} \]
The first two enzymes of pyrimidine biosynthesis, are fused to form a CPSase-ATCase complex with an inactive DHOase in between (119). Interestingly, the molecular mass of the CPSase-ATCase complex of *S. cerevisiae* is similar to the molecular mass seen in CAD which has three functional proteins. Upon sequence analysis, it was shown that *S. cerevisiae* contains a region of homology between CPSase and ATCase that is similar to known DHOases. However, this open-reading frame did not encode an active DHOase (119).

Bacterial ATCases have been divided into three classes depending on their molecular mass, subunit composition, and allosteric properties (8). Schematic representations of the three bacterial ATCase classes are shown in Figure 3. Class C ATCases have the smallest molecular mass of 100 kDa and are composed of three identical polypeptides of Mr 34 kDa. The class C ATCases exhibit Michaelis-Menten kinetics for both substrates, aspartate and carbamoylphosphate, and are not regulated by nucleotide effectors such as ATP, CTP, or UTP (10). Gram positive bacteria such as *Bacillus subtilis* (10), *Enterococcus faecalis* (17), and *Staphylococcus epidermidis* (63), have trimeric ATCase of Mr 100 kDa with polypeptide subunit Mr of 34 kDa. Recent studies from Shepherdson’s laboratory have shown that some Gram negative bacteria such as *Xanthomonas campestris*, *Stenotrophomonas maltophilia*, and *Lysobacter enzymogenes* also have trimeric ATCases with molecular masses of 100 kDa with a polypeptide Mr of 34 kDa.
FIGURE 3. Bacterial ATCases

Class A ATCases
\( M_r \approx 480 \text{ kDa} \)

One Polypeptide of Catalytic Trimer
34 kDa
\( (pyrB) \)

45 kDa \( (pyrC') \)

Class B ATCases
\( M_r \approx 310 \text{ kDa} \)

One Polypeptide of Catalytic Trimer
34 kDa
\( (pyrB) \)

Regulatory Dimer
17 kDa
\( (pyrI) \)

Class C ATCases
\( M_r \approx 100 \text{ kDa} \)

One Polypeptide of Catalytic Trimer
34 kDa
\( (pyrB) \)
The catalytic trimer of *E. coli* is also classified as class C ATCase. In all organisms listed in the class C ATCases, the catalytic activity is associated with a polypeptide of 34 kDa which forms a trimeric structure.

Class B ATCases, which have molecular masses of approximately 310 kDa, are found in the enteric bacteria with the *E. coli* ATCase being the best studied (36, 38, 50, 59, 107). The holoenzyme of *E. coli* has a molecular mass of 310 kDa and is composed of two different proteins: a catalytic polypeptide encoded by *pyrB* and a regulatory polypeptide encoded by *pyrI* (105). Six identical polypeptide chains form two trimeric catalytic subunits and six identical polypeptide chains form three dimeric regulatory subunits with a dodecameric architecture of 2c3:3r2. The catalytic polypeptide has an Mr of 34 kDa contains two domains. A carbamoylphosphate binding region is found in the polar domain (amino terminus) and an aspartate binding region is located in the equatorial domain (carboxy terminus). Likewise, the regulatory polypeptide with a Mr of 17 kDa (131) has two domains, the effector binding sites for ATP and CTP are housed at the amino terminus while the carboxy terminus contains the zinc binding sites. Six zinc molecules stabilize the regulatory dimer and promote the association of regulatory and catalytic subunits (80, 89, 106, 132). A schematic representation of the quaternary structure of the class B ATCase is shown in Figure 4 (65).
FIGURE 4. Quaternary Structure of ATCase

C=Catalytic subunit, R=Regulatory subunit, Asp=Aspartate Binding Site, CP=Carbamoylphosphate Binding Site, Zn=Zinc Domain, AL=Allosteric (Nucleotide) Binding Site
The ATCase holoenzyme in *E. coli* exhibits sigmoidal kinetics with both aspartate and carbamoylphosphate when initial velocity is plotted as a function of substrate concentration (8). When velocity-substrate plots are carried out using the catalytic subunits only, a hyperbolic or Michaelis-Menten kinetic curve is observed using either substrate. Michaelis-Menten kinetics are observed also if the assay temperature were lowered to 4°C in the presence or absence of CTP (91) or if the native enzyme were treated with 0.1 mM urea or assayed at pH 8.0 (132).

Yates and Pardee (140) showed that in *E. coli*, ATCase is feedback inhibited by CTP and activated by ATP. Yet, the inhibition of ATCase by CTP can be overcome by increasing aspartate concentrations to saturating levels of 25 mM or greater. ATP decreases the $K_m$ of ATCase for aspartate whereas, CTP increases the $K_m$. UTP by itself does not inhibit the activity of ATCase, but in concert with CTP, a synergistic inhibition is seen which is not seen when UTP or CTP is used separately (135, 136, 143).

The regulatory and catalytic subunits of class B ATCases can be dissociated by treating the holoenzyme with heat or mercurial compounds such as *p*-hydroxymercuribenzoate or neohydrin (37, 39). The catalytic trimer remains enzymatically active, but there is a loss of nucleotide effector response. Moreover, the separated catalytic trimer appears to be four times more enzymatically active than the holoenzyme (60).
These active dissociated subunits can be reassembled to form
the holoenzyme with no loss of catalytic or regulatory activity
when incubated in β-mercaptoethanol or dithiothreitol (37, 39).

Class A ATCases have the largest molecular mass of between
450 and 500 kDa and are mostly found in the pseudomonads (1, 7,
63, 110, 111, 128). The first class A ATCase was purified from
P. fluorescens (1) and was thought to be composed of two
identical subunits of Mr of 180 KDa. The class A ATCases were
considered to be dimers until Bergh and Evans (7) showed that
the P. fluorescens ATCase enzyme was actually composed of two
polypeptide chains with molecular masses of 34 kDa and 45 kDa.
The 34 kDa chain, encoded by pyrB, contains both the active
site for ATCase and the nucleotide effector binding sites. The
34 kDa polypeptide is the same size as the catalytic
counterparts found in other bacterial (8, 10, 18, 39, 60, 63,
125), fungal (119), and CAD (47, 87, 108) aspartate
transcarbamoylases. This 34 kDa polypeptide is universal in
ATCases studied thus far.

Bergh and Evans (7) concluded that catalytic and
regulatory domains were both located on the 34 kDa polypeptide
using affinity labelling with an analog of the allosteric
effector ATP. This point was verified by Alan Kumar who
deleted the first 34 amino acids from the amino terminus of the
ATCase of P. putida and showed that while activity was retained
there was no nucleotide inhibition (66).
The 45 kDa chain is encoded by pyrC' and produces an inactive DHOase-like polypeptide. It has been designated pyrC' because of the striking sequence homology to known dihydroorotases, yet lacks DHOase activity, by virtue of its missing five essential histidine residues (110, 111). Purified ATCase did not contain any DHOase activity which could be seen in nearby fractions from sonicates of the purification process (Figure 5).

FIGURE 5. Representation of Pyrimidine Biosynthetic Enzymes Passed over a Size-Exclusion Column
The pyrC' gene, isolated on a plasmid, expressed in E. coli did not complement an E. coli pyrC mutant, suggesting that the pyrC' did not encode the functional DHOase. The true DHOase gene, pyrC, was cloned from a P. aeruginosa cosmid library and was shown to reside elsewhere on the P. aeruginosa chromosome (12). In P. putida and P. aeruginosa, there is a four base pair overlap between the pyrB and pyrC', which insures that each polypeptide will be produced in equimolar amounts (142). At the present time, the 45 kDa chain is thought to be needed to maintain dodecameric architectural integrity.

The class A ATCases exhibit Michaelis-Menten saturation curves with both aspartate and carbamoylphosphate when initial velocity is plotted as a function of substrate concentration. In both P. aeruginosa and P. putida inhibition is seen with nucleotide effectors ATP, CTP, and UTP, but the amount of inhibition varies considerably for the two pseudomonad species (110, 111, 129).
CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains. The bacterial strains used in this study were obtained from the American Type Collection Culture Collection (ATCC, Rockville, MD.). Bacterial strains, ATCC number, optimal growth temperature, and growth requirements are listed in Table 2 on the following page. Long term storage of bacterial strains was achieved by inoculating five to six well-isolated colonies from an agar plate having 18 - 24 hours growth into a Pro-Lab cryovial. The vial was then inverted several times to mix the organism with porous beads and cryopreservative. The cryopreservative was then removed with a sterile Pasteur pipette and the cryovial stored at -20°C. When a new culture was needed, a bead was removed from the vial and used to inoculate a broth or agar plate. With the exception of Brevundimonas diminuta, which was kept at room temperature and inoculated on a nutrient agar plate, all working strains were kept at 4°C and were streaked for isolation on Pseudomonas minimal agar medium. Both Shewanella putrefaciens and Stenotrophomonas maltophilia required that 0.2 ml of 10% casamino acids be spread on Pseudomonas minimal agar plates for growth. Most strains were viable for four to six weeks on agar plates, but Burkholderia cepacia,
TABLE 2. Bacterial Strains

<table>
<thead>
<tr>
<th>Organism and ATCC Number</th>
<th>Optimal Temperature and Growth Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 10145</td>
<td></td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 13525</td>
<td></td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 13985</td>
<td></td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 12633</td>
<td></td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 19310</td>
<td></td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 17588</td>
<td></td>
</tr>
<tr>
<td><em>P. mendocina</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 25411</td>
<td></td>
</tr>
<tr>
<td><em>P. pseudoalcaligenes</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 17440</td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 25416</td>
<td></td>
</tr>
<tr>
<td><em>B. pickettii</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 27511</td>
<td></td>
</tr>
<tr>
<td><em>Comamonas acidovorans</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 15668</td>
<td></td>
</tr>
<tr>
<td><em>C. testosteroni</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 11996</td>
<td></td>
</tr>
<tr>
<td><em>Brevundimonas diminuta</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 11568</td>
<td>Pantothenate</td>
</tr>
<tr>
<td></td>
<td>Biotin</td>
</tr>
<tr>
<td></td>
<td>Cystine</td>
</tr>
<tr>
<td></td>
<td>Cyanocobalamine</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 13637</td>
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</tr>
<tr>
<td><em>Pseudomonas indigofera</em></td>
<td>37°C</td>
</tr>
<tr>
<td>ATCC 14036</td>
<td></td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em></td>
<td>30°C</td>
</tr>
<tr>
<td>ATCC 8071</td>
<td></td>
</tr>
</tbody>
</table>
*Burkholderia pickettii*, *Shewanella putrefaciens*, and *Stenotrophomonas maltophilia* needed to be subcultured once a week to keep viable on agar plates (or a cryobead could be used when a new culture was needed).

**Chemicals and Reagents.** Antipyrine, ammonium persulfate, L-aspartate (monopotassium salt), biotin, carbamoylaspartate (CAA), cyanocobalamine, disodium salts of ATP, CTP, and UTP, L-histidine, nitrilotriacetic acid, pantothenate, sodium sulfide, succinic acid (disodium salt hexahydrate), and Trizma base were purchased from Sigma Chemical Co. Bromophenol blue, glycerol, methanol, potassium acetate, sucrose, sulfuric acid, and zinc sulfate were purchased from J.T. Baker Co. Ultra pure ammonium sulfate was purchased from Bethesda Research Lab (BRL). Sephacryl was purchased from Pharmacia Biotech. Sodium sulfite was purchased from Kodak Co. The following items ordered from Bio-Rad were of electrophoresis purity: acrylamide, bis-acrylamide, Coomassie brilliant blue, glycine, TEMED (NNN',N' tetramethylethlenediamine), and sodium dodecyl sulfate (SDS). Acetic acid, agar, ammonium acetate, ammonium sulfate, ammonium molybdate, blue dextran, β-mercaptoethanol, 2,3 butanedione monoxime, cobalt nitrate, cupric sulfate, dextrose, dibasic sodium phosphate, diethanolamine, dipotassium phosphate, disodium ethylenediamine tetraacetate (EDTA), ferric sulfate, Folin reagent, lead nitrate, magnesium acetate, magnesium chloride, magnesium sulfate, N-
ethylmorphine, 2 [N morpholin]ethanesulfonic acid (MES), potassium hydroxide, potassium phosphate, sodium borate, sodium chloride, sodium carbonate, sodium citrate, and sodium/potassium tartrate were purchased from Fisher Scientific Co.

**Growth Media.** All pseudomonads and "former" pseudomonads were grown in *Pseudomonas* minimal medium (PMM) (88). A liter of PMM was prepared by adding 25 ml 0.5 M Na$_2$HPO$_4$, 25 ml 0.5 M KH$_2$PO$_4$, 10 ml 10% (NH$_4$)$_2$SO$_4$, 10 ml concentrated base (recipe to follow), to 930 ml of distilled deionized water (ddH$_2$O) in a 2.8 l Fernbach flask. The solution was autoclaved for 40 minutes at 121°C. After the solution had cooled, 10 to 50 mM succinate was added. If agar plates were required, the main ingredients listed above were brought up to a 500 ml volume with ddH$_2$O and placed in a 2 l Erlenmeyer flask. In a separate 2 l Erlenmeyer flask, 15 g (final volume 1.5%) of agar were mixed with the remaining 430 ml of ddH$_2$O. The solutions were autoclaved as above. After the two flasks were slightly cooled, the contents of the first flask were poured into the second flask containing the agar mixture, 10 mM succinate (final concentration) was added to the flask before the agar plates or slants were poured.

Certain strains in this study required additional growth factors when the organism was grown in PMM. *Stenotrophomonas maltophilia* and *Shewanella putrefaciens* required 10 ml (10%) casamino acids be added to one liter of PMM. *Brevundimonas*
diminuta required 10 ml of 10% casamino acids, pantothenate (0.4 mg), biotin (0.4 mg), and cyanocobalamin (0.4 mg) be added per one liter of PMM.

One liter of stock concentrated base used for PMM was prepared by dissolving 20 g nitrilotriacetic acid in 600 ml ddH₂O and neutralizing it with 14.6 g KOH. All components must be completely dissolved before adding the next ingredient and ingredients must be added in the given order. 28.9 g MgSO₄ anhydrous, 6.67 g CaCl₂•7H₂O, 18.5 mg (NH₄)₆Mo₇O₂₄•7H₂O, 198.0 mg FeSO₄•H₂O, and 100 ml metals 44 (recipe to follow) were added to the nitrilotriacetic acid solution and the pH was adjusted to 6.8 before bring the solution up to a volume of 1 liter. The solution should be a light yellow color. The concentrated base can either be autoclaved or overlayed with toluene for long-term storage at 4°C.

One liter of Metals 44 solution was prepared by dissolving the following ingredients in the given order. To 800 ml ddH₂O add 2.5 g EDTA, 10.95 g ZnSO₄•7H₂O, 5.0 g FeSO₄•7H₂O, 1.54 g MnSO₄•H₂O, 392.0 mg CuSO₄•5H₂O, Co(NL₃)₂•6H₂O, and 177.0 mg Na₂B₄O₇•10H₂O. The solution should be brought to one liter volume with ddH₂O and have a lime green appearance. A few drops of concentrated H₂SO₄ was added to the solution to prevent precipitation. This medium could be stored indefinitely at room temperature.
One liter of *E. coli* minimal medium (ECMM) (85) was prepared by adding 10.5 g K$_2$HPO$_4$, 4.5 g KH$_2$PO$_4$, 1.0 g (NH$_4$)$_2$SO$_4$, and 0.5 g Na$_3$Citrate to a 2.8 l Fernbach flask containing 950 ml ddH$_2$O. The ECMM was the autoclaved for 40 minutes at 121°C. After the solution cooled, one ml of sterile MgSO$_4$ at 1.0, one ml of sterile 1000X thiamin, and 10 ml of sterile 20% dextrose were added.

One liter of nutrient agar plate medium was made by dissolving 23 g of Difco nutrient agar with one liter ddH$_2$O into a 2 l Erlenmeyer flask and autoclaving the mixture for 40 minutes at 121°C. After the agar mixture cooled, 25 to 30 ml was dispensed to each Petri plate.

**Harvesting of Bacterial Strains.** All strains were first inoculated into a flask containing 50 ml of PMM or ECMM and grown overnight at their appropriate temperature in a shaker incubator. After 18 to 24 hours of growth, the 50 ml flask was used to inoculate a 2 or 4 liter flask containing either one or two 1 of PMM or ECMM, which was then incubated for 18 to 24 hours. Typically an absorbance of 1.5 at 260 nm was obtained. The cells were then pelleted by centrifugation at 8,000 xg for 20 minutes at 4°C using a Sorvall R5C5 centrifuge with a GS3 rotor. The wet weight of the pellet for each organism was recorded, as well as initial volume of growth medium. Pellets were stored in a 50 ml conical tube and kept at -20°C until needed.
Preparation of Cell Extracts. Pellets that were stored at -20°C were thawed and resuspended in 2 ml ATCase breaking buffer (50 mM Tris, 2.0 mM mercaptoethanol, and 20 mM ZnSO₄) for each gram of wet weight and sonicated in an ethanol ice bath for 15 minutes using a Branson Cell Disruptor 200. The cell slurry was then centrifuged at 15,000 x g for one hour at 4°C using a Sorvall RC5C centrifuge with a SA600 rotor. The supernatant typically was then transferred to a 15 ml or 50 ml conical tube and stored at 4°C. A 0.3 ml sample from the supernatant of each organism was kept at 4°C for later determination of ATCase activity and protein concentration.

Pilot Purification of ATCase. ATCase from each organism was partially purified to be used later for kinetic studies. All steps of purification were carried out at 4°C. A streptomycin sulfate solution was made by mixing 10% (w/v) streptomycin sulfate in a 0.1 M phosphate buffer (pH 7.5). An equal volume of this 10% streptomycin sulfate solution was added slowly to a known volume of cell extract. After slowly mixing the two solutions for one hour, the debris was pelleted by centrifugation for one hour at 16,000 x g. A 0.3 ml sample of the supernatant was removed for later ATCase and protein analysis. The remaining supernatant was measured and used in the next purification step.

The next step of purification involved the use of two ammonium sulfate precipitations. First, the correct ammonium sulfate cuts had to determined for each organism. The cuts
were determined by first adding ammonium sulfate (20% final concentration) to a 10 ml volume of each cell extract and stirring for 30 minutes. The ammonium sulfate treated cell extract was centrifuged for 30 minutes at 15,000 xg. A 0.1 ml sample of the supernatant was removed for later ATCase determination and the rest of the supernatant was used for the next ammonium sulfate cut. The pellet that remained after the supernatant was removed was then resuspended in 0.1 ml of ATCase breaking buffer. This was saved for ATCase determination also. The supernatant was measured to determine the amount of ammonium sulfate to be added for the next cut (5% higher that the previous cut). These steps were repeated until the ammonium sulfate final concentration reached 65%. At this time, ATCase assays were performed (assay described later) on each supernatant and pellet samples to determine what ammonium sulfate cuts were to be used.

During the first ammonium sulfate cut, ATCase was found mostly in the supernatant and after the second ammonium sulfate cut, ATCase was found in the pellet (Table 3). **Final Purification of ATCase.** For the first cut ammonium sulfate was added slowly to 25 ml cell extract and stirred for one hour. The solution was then centrifuged for one hour at 15,000 x g. The supernatant was removed and the volume was recorded. A 0.3 ml sample was removed from the
TABLE 3. Ammonium Sulfate Cuts

<table>
<thead>
<tr>
<th>Organism</th>
<th>(NH$_4$)$_2$SO$_4$ First Cut</th>
<th>(NH$_4$)$_2$SO$_4$ Second Cut</th>
<th>(NH$_4$)$_2$SO$_4$ mg/ml</th>
<th>(NH$_4$)$_2$SO$_4$ mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>P. aureofaciens</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>P. putida</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>P. syringae</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>P. stutzeri</td>
<td>25%</td>
<td>40%</td>
<td>0.134</td>
<td>0.084</td>
</tr>
<tr>
<td>P. mendocina</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>P. pseudoalcaligenes</td>
<td>30%</td>
<td>45%</td>
<td>0.164</td>
<td>0.086</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>25%</td>
<td>40%</td>
<td>0.134</td>
<td>0.084</td>
</tr>
<tr>
<td>B. pickettii</td>
<td>25%</td>
<td>45%</td>
<td>0.134</td>
<td>0.115</td>
</tr>
<tr>
<td>Comamonas acidovorans</td>
<td>20%</td>
<td>50%</td>
<td>0.106</td>
<td>0.175</td>
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<tr>
<td>C. testosteroni</td>
<td>20%</td>
<td>50%</td>
<td>0.106</td>
<td>0.175</td>
</tr>
<tr>
<td>Brevundimonas diminuta</td>
<td>20%</td>
<td>50%</td>
<td>0.106</td>
<td>0.175</td>
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<tr>
<td>Stenotrophomonas maltophilia</td>
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<td>55%</td>
<td>0.106</td>
<td>0.207</td>
</tr>
<tr>
<td>Pseudomonas indigofera</td>
<td>25%</td>
<td>45%</td>
<td>0.134</td>
<td>0.115</td>
</tr>
<tr>
<td>Shewanella putrefaciens</td>
<td>20%</td>
<td>60%</td>
<td>0.106</td>
<td>0.241</td>
</tr>
</tbody>
</table>
supernatant and stored at 4°C for later ATCase activity and protein determinations. For the second cut, ammonium sulfate was slowly added to the supernatant and stirred for three hours. The mixture was then centrifuged for one hour at 15,000 x g. The supernatant was removed and stored in a 50 ml conical tube and kept at 4°C. The pellet was resuspended in four ml ATCase breaking buffer. A 0.3 ml sample was removed and stored at 4°C for later ATCase activity and protein determination. At this point, the samples could be stored at 4°C for months and still retain original ATCase activity.

The final step of purification involved passage of the ammonium sulfate treated cell extract over a Pharmacia XK26 (70 cm) column using Sephacryl 400S. The column buffer consisted of 10 mM Tris pH 8.2, 1 mM EDTA, 20 mM zinc acetate, 0.5 mM β-mercaptoethanol and 150 mM potassium acetate per liter of ddH2O. First a tracking dye of 0.1 g blue dextran and 2 ml ddH2O was loaded unto the column, which was followed by loading a three ml sample onto the column using a Pharmacia peristaltic pump P1. When the blue dye reached the end of the column, 5 ml fractions (100 drops) were collected using a Bromma 2212 Helirac Fraction Collector. Each fraction was then assayed for ATCase activity. The tube having the highest ATCase activity was then used for further kinetic studies.
The ATCase from *Burkholderia cepacia* was further purified by linear gradient elution through a Waters Protein-Pak DEAE HR8 anion exchange column run on a Waters high-performance liquid chromatography (HPLC) system. The gradient was established by running 100% buffer A (20 mM Tris [pH 8.2]) to 75% buffer B (20 mM Tris [pH 8.2], 1M NaCl) over a period of 40 minutes at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and used for further studies.

ATCase activity and protein concentration from each step of the purification were determined and used to calculate specific activity, fold-purification, and yield. Specific activity for ATCase was defined as μmol of carbamoylaspartate produced per minute per mg of protein. Fold-purification was determined by dividing the specific activity of the purification step by the original specific activity of the cell extract. Yield was determined by dividing the total units (volume x optical density at 466 nm) of the purification step by the original total unit of the cell extract.

**ATCase Assay.** Cell extracts or partially purified cell sonicates were assayed for ATCase activity by measuring the amount of carbamoylaspartate (CAA) produced at 10, 20, and 30 minute intervals at 30°C (38) with a modified color reaction (101). ATCase assays were performed to determine optimal pH, Vmax, and Km varying either aspartate or carbamoylphosphate using either aspartate or carbamoylphosphate as substrate.
with the other substrate in excess at saturation. A "tribuffer system" (0.05 M MES, 0.1 M diethanolamine, and 0.051 M diethanolamine) (29) with pH ranging from 7.0 to 10.0 was used to determine the optimal pH for the ATCase of each organism. With the exception of Brevundimonas diminuta with an optimal pH of 9.0, all organisms in this study had an optimal pH of 9.5 which was used throughout the study. All assay reaction tubes were prepared in advance and stored on ice until needed. When aspartate was the substrate varied, the assay tubes contained the following reagents in a final volume of 2 ml: 0.08 ml Tribuffer pH 9.5 or 9.0 (0.051 M MES, 0.1 M diethanolamine, 0.051 M N-ethylmorpholine), 0.2 ml aspartate (final concentration 0.5 mM - 20 mM), 0.04 ml diluted enzyme sample, and ddH2O to volume. If carbamoylphosphate were used as the substrate varied, 0.2 ml of the saturating aspartate was used in each reaction tube and the reaction was initiated by adding varying concentrations of carbamoylphosphate (final concentration 0.081 mM - 10.4 mM). Effector response could be measured by adding ATP, CTP, or UTP (final concentration 1 mM) to the assay reaction tubes. Control tubes to check background color development of substrates, buffer, and enzyme were also assayed. A blank control tube final volume 2 ml was made by adding all ingredients, but the enzyme was omitted. Two substrate control tubes were made by adding all ingredients
in a final volume of 2 ml and omitting either aspartate or carbamoylphosphate for each substrate control tube.

The assay reaction tubes were preincubated at 30°C for 5 minutes. The reaction was initiated by the addition of 0.2 ml of carbamoylphosphate (8 mg/ml) at 15 second intervals. At 10, 20, or 30 minute timepoints, 0.5 ml of the reaction assay mixture was transferred to a color reaction tube containing 0.5 ml ddH₂O and 1 ml color mix (2 parts antipyrine (5 mg/ml) in 50 % sulfuric acid and 1 part monoxime (8 mg/ml) in 5 % acetic acid) which were stored on ice. After transfer of 0.5 ml assay mixture to the color mix reaction tube, a marble was placed on the color reaction tube. This was an easy way to visualize what tube to use next and served to prevent evaporation during color development. When all assay reaction mixtures had been added to the color reaction tubes, the color reaction tubes were placed in a 60°C water bath, exposed to room light, and incubated for two hours for color development. After the two hour incubation, the tubes were removed from the water bath and allowed to cool to room temperature in a dark place. In a Perkin-Elmer Lambda 3A UV/Vis spectrophotometer absorbances were read at 466 nm. Velocity-substrate curves were generated by plotting the specific activity of the enzyme (nmol carbamoylaspartate per minute per mg of protein) versus the concentration of either aspartate or carbamoylphosphate. The Km for both aspartate and
carbamoylphosphate were determined from the intercepts of the x-axis using a Lineweaver-Burk plot (1/substrate versus 1/velocity). The total amount of protein from each extract was quantified by the method of Lowry (75). Reconstituted bovine serum albumin (1% stock solution) was used as the standard.

**ATCase Activity Polyacrylamide Gel.** Bothwell originally developed the polyacrylamide ATCase activity stain to show the architectural forms of purified subunits (9). This method was later modified so that ATCase architectural structures could be detected using crude cell extracts (61). A 6% nondenaturing polyacrylamide gel was prepared by mixing 9.64 ml of stock acrylamide (40% acrylamide: 1% bisacrylamide), 7.5 ml separating gel buffer (36.3 g Tris (pH 8.9) up to 100 ml ddH2O), 4.48 ml 40% sucrose which was brought up to a 60 ml volume with ddH2O. Before degassing the acrylamide solution, 0.5 g ammonium persulfate was added. After the acrylamide solution was degassed, 0.04 ml TEMED was added to the solution which was then poured into a Hoefer SE 400 vertical slab gel unit with a 10 well comb inserted. Diluted sample were mixed 1:1 with loading dye (1 ml separating gel buffer - 1M Tris pH 8.9, 1 ml glycerol, and 1 ml ddH2O with 0.25 mg bromophenol blue). A total volume of 20 μl of 1:1 sample/loading dye was loaded into each individual well. Partially purified ATCase from E. coli (both holoenzyme and catalytic trimer) and P. aeruginosa with known molecular mass
was used as controls. The molecular mass of ATCases used in this study were determined by comparing the sample banding pattern to that of known ATCase standard markers. *E. coli* has a molecular mass of 310 kDa and 100 kDa and *P. aeruginosa* has a molecular mass of 480 kDa. After the samples had been loaded, the gel was electrophoresed for 9 hours at 4°C, and 5 watts. The electrode buffer consisted of 10 mM Tris (pH 8.3) and 40 mM glycine up to a final volume 2 liters with ddH2O.

After electrophoresis, the acrylamide gel was placed in a pyrex dish containing 250 ml ice-cold 50 mM histidine (pH 7.0) for at least 5 minutes. The reactants, 5 ml 1 M aspartate and 10 ml 0.1 M carbamoyl aspartate, were then added to the pyrex dish. During this 10 minute time period, ATCase was catalyzing the reaction of aspartate and carbamoylphosphate to form carbamoylphosphate which released orthophosphate in the process. The gel was rinsed three times with ice-cold distilled water to remove reactants. The orthophosphate was captured by adding 250 ml of 3 mM lead nitrate in ice-cold 50 mM histidine (pH 7) to the Pyrex dish containing the gel. The gel was gently rocked for 10 minutes and then rinsed with 3 changes of ice-cold water. ATCase bands appear as white precipitate bands. Intensity of these bands was increased after storage at 4°C.

Photographs of ATCase activity gels were obtained by placing the gel on a flat surface having a black or dark background. Using one set of flood light, no filter, and a
camera with an aperture setting of f/16, a Polaroid type 55 positive/negative film was exposed for 10 seconds. After a 30 second development, the positive picture was separated from the negative. The negative was then rinsed in a 9% sodium sulfite solution until clear. It was then rinsed with distilled water and allowed to dry. The negative could then be used to make additional prints as needed.

**Electroelution of Large and Small ATCase Bands of* Burkholderia cepacia *from Polyacrylamide Gel.**  
A 6% non-denaturing polyacrylamide gel, prepared as before, was poured using an inverted well comb to create one oblong well 3/4 inches deep and 5 inches long. One ml of partially purified *B. cepacia* cell extract was mixed with 0.1 ml 10X non-denaturing loading dye and loaded into the one large well (Figure 6). The gel was then electrophoresed at 4°C for 9 hours at 5 watts. Upon removal of the gel apparatus, the gel was placed in a Pyrex dish with distilled water. A one inch segment was cut from the left side of the gel and was used to stain for ATCase activity. After the staining procedure was completed, the one inch segment was realigned with the gel and used to visualize what part of the gel should be cut out. The large ATCase band of *B. cepacia* was cut out with a razor blade and placed onto a small piece of plastic wrap. Likewise, the small ATCase band of *B. cepacia* was cut out and placed onto another small piece of plastic wrap. Each band was slightly minced and placed into a 15 ml
FIGURE 6. Isolation of Large and Small ATCases from *Burkholderia cepacia*

After electrophoresis, a small segment was cut and stained for ATCase activity, the segment was then realigned, and the separate ATCase bands were removed for electroelution.
conical tube. Enough ATCase buffer was added to each conical tube to cover the amount of minced acrylamide containing the enzyme and stored at 4°C until ready for use. This procedure was repeated for each ml of *B. cepacia* cell extract. When all of the *B. cepacia* extract was used, the individual minced bands along with the ATCase buffer that was used for storage were placed in separate dialysis tubes. The dialysis tubes containing the minced ATCase band, were then electroeluted at 4°C for 2 hours at 5 watts using the activity gel electrode buffer. A Pasteur pipette was used to remove the electroeluted ATCase from the dialysis tube. It was placed into separate 15 ml plastic conical tubes and stored at 4°C until ready for use.

The electroeluted samples from both the large and small ATCase bands of *B. cepacia* were then electrophoresed a second time in a 6 % non-denaturing polyacrylamide gel having 5 wells or lanes (Figure 7). The first two wells were loaded with a mixture of 0.5 ml of the large electroeluted *Burkholderia cepacia* ATCase and 0.05 ml of 10X non-denaturing loading dye. The center lane was void of sample and the last two wells contained the same volume of the small electroeluted *B. cepacia* ATCase. The gel was then electrophoresed at 4°C for 9 hours at 5 watts. The outer two lanes were cut and stained for ATCase activity and then realigned so that the bands from lanes 2 and 4 could be
FIGURE 7. Electroelution of Large and Small ATCases from *Burkholderia cepacia*

After electrophoresis, the outer band were cut and stained for ATCase activity.

The stained well were then realigned, and the band cut from wells 2 & 4.
removed. As before, these acrylamide gel fragments were cut from the gel and slightly minced before placing them into separate dialysis tubes that contained 0.5 ml to 1 ml of ATCase buffer. They were electroeluted at 4°C for 2 hours at 5 watts using the activity gel electrode buffer. The purity of ATCase was greatly enhanced by repeating the electrophoresis followed by electroelution. The electroeluted sample was removed from the dialysis tubes and concentrated for further studies; such as, molecular weights of individual polypeptides and kinetic studies.

**Concentration of ATCase Samples.** Microcon-10 microconcentrators were used as a fast and efficient means for concentrating ATCase. A microcon sample reservoir was first inserted into a microcentrifuge vial. A maximal volume of 0.5 ml of sample was pipetted into the sample reservoir. The sample was then centrifuged in a microfuge tube at 4°C for 15 minutes at 14,000 x g. The sample reservoir was removed and inverted onto another microcentrifuge vial which was then microcentrifuged for 3 minutes at 1,000 x g. The concentrated ATCase could be stored in the microcentrifuge vial at 4°C until needed.

**Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis.** A 12% denaturing SDS polyacrylamide gel was electrophoresed to determine ATCase polypeptide molecular weights. A 12% SDS separating gel was prepared by mixing 4 ml acrylamide stock solution (30% acrylamide:0.8% bis-
acrylamide), 2.5 ml 4X separating gel buffer (75 ml 2 M Tris-HCl (pH 8.8), 4 ml 10 % SDS, and 21 ml ddH2O), and 3.5 ml ddH2O. Prior to pouring the gel, 0.02 g of ammonium persulfate was added to the separating gel mixture and gently mixed, followed by the addition of 5 µl of TEMED. The separating gel solution was poured into a BCL120 mini vertical gel system apparatus, leaving a 3 cm gap at the top. Butanol was then layered over the separating gel until the separating gel solution polymerized. After polymerization, the butanol was removed and the gap area rinsed well with distilled water. A stacking gel was then poured for the remaining volume. The stacking gel was prepared by mixing 0.67 ml 30 % acrylamide, 1 ml 4X stacking gel buffer (50 ml 1 M Tris-HCl (pH 6.8), 4 ml 10 % SDS, and 46 ml ddH2O), and 2.3 ml ddH2O. Prior to pouring, 0.02 g of ammonium persulfate was added to the stacking gel mixture and gently mixed, followed by the addition of 5 µl of TEMED. A 10 well comb was then inserted and the stacking gel allowed to polymerize. After polymerization the gel was placed in the mini gel buffer chamber containing SDS electrode buffer (3 g Tris pH 8.2, 14.4 g glycine, 1 g SDS, brought up to a 1 liter volume with ddH2O).

A volume of 15 µl of sample was mixed with 3 µl 5X sample buffer (0.6 ml Tris-HCl (pH 6.8), 5 ml 50 % glycerol, 2 ml 10 % SDS, 0.5 ml 2-mercaptoethanol, 1 ml 1% bromophenol blue, and 0.9 ml ddH2O). The sample buffer was stable for
several months when stored at 4°C. Each 20 μl sample mixture along with a 5 μl Promega mid-range molecular weight marker was boiled 5 minutes before loading into the SDS gel. After the gel was loaded, the gel was electrophoresed at room temperature for 1.5 hours at 200 volts.

The SDS polyacrylamide gel was then stained for 5 minutes with Coomassie Blue stain. A 1 liter stock of Coomassie Blue stain was prepared by mixing 1.0 g Coomassie Blue, 450 ml methanol, 450 ml ddH₂O, and 100 ml glacial acetic acid. After gently rocking the gel in the Coomassie Blue stain, the gel was destained overnight. The destaining stock consisted of 100 ml methanol, 100 ml glacial acetic acid, and 800 ml ddH₂O.

For low protein concentrations that the Coomassie Blue stain could not detect, a silver stain was used to stain over the already stained SDS polyacrylamide gel. Silver stain has the highest sensitivity, having a 100-fold increase in sensitivity over the Coomassie Blue stain, being able to detect as low as 0.02 ng/mm² (81). A Gelcode silver stain kit purchased by Pierce was used to stain the SDS polyacrylamide gel. Working reagents were diluted from concentrated stock reagents immediately before use. The gel was placed in the Silver solution and gently rocked for 30 minutes. The gel was then rinsed with water for one minute. The gel was then placed in a reducer solution for 5 minutes. The gel was
rinsed again with water. The gel was then placed in a stabilizer solution and allowed to gently rock for 30 minutes after which the gel could be stored in water or wrapped in clear plastic wrap.

Molecular weights were then determined by comparing the sample bands with those of the known Promega mid-range molecular weight marker. The Promega mid-range molecular weight marker had the following molecular weight markers: 97.4 kDa, 66.2 kDa, 55.0 kDa, 42.7 kDa, 40.0 kDa, 31 kDa, 21.5 kDa, and 14.3 kDa.
Class A aspartate transcarbamoylases (ATCases) are the largest of the three reported classes of ATCases (8) and are found in the fluorescent pseudomonads (110, 111, 129). They have molecular masses of about 480 kDa and like their class B (34) and class C (10) counterparts contain a trimer of three identical polypeptides, each of approximately 34 kDa. In addition to the 34 kDa polypeptides, the Pseudomonas holoenzymes contain another polypeptide of approximately 45 kDa molecular mass. Stoichiometric calculations suggest that six 34 kDa catalytic polypeptides associate with six 45 kDa polypeptides to form an active dodecamer of molecular mass, 480 kDa. Such a structure maintains the enteric-type dodecameric architecture where two catalytic trimers 2(34 x 3) combine with three 45 kDa dimers 3(45 x 2) to form the prototype holoenzyme. Unlike the enteric enzyme where the catalytic homotrimer is fully active, the trimer in Pseudomonas has no activity.

Moreover, derived amino acid sequence comparisons have shown that the 45 kDa polypeptide is a dihydroorotase (DEOase)-like polypeptide, which itself has no DEOase
activity (110, 111). It is suggested that the DHOase-like polypeptides have been recruited to maintain the dodecameric structure that is required for the ATCase activity in the pseudomonads. The two genes encoding the 34 kDa polypeptide, \textit{pyrB}, and the 45 kDa polypeptide, \textit{pyrC'}, have been cloned and sequenced in \textit{P. putida} (110, 111) and in \textit{P. aeruginosa} (129). The \textit{pyrBC'} genes comprise an operon wherein the \textit{pyrC'} gene overlaps the \textit{pyrB} gene by 4 base pairs. Such an overlap is believed to insure equimolar amounts of both \textit{pyrB} and \textit{pyrC'} polypeptides and affords the retention of the inactive DHOase gene even though a fully active \textit{pyrC} exists elsewhere on the \textit{Pseudomonas} chromosome (12).

The research presented in this dissertation was initiated for three reasons. 1) The unusual nature of the ATCase first from \textit{P. putida} and later from \textit{P. aeruginosa} suggests that a wider distribution of the class A ATCases was likely. Because of two earlier studies from O'Donovan's Laboratory (110, 111, 129) it was thought that the \textit{Pseudomonas} ATCase could be used as a taxonomic marker. 2) The state of flux of the \textit{Pseudomonas} genus could now be further investigated if the ATCase from those retained \textit{Pseudomonas} species were examined and compared to the ATCases from newly assigned species. Thus, changes already made could be validated and further changes suggested. 3) The third and most compelling reason for doing this particular research derives from my 12-year clinical microbiology career
at Children's Medical Center of Dallas, where I have been intimately associated with *Pseudomonas* species. *Pseudomonas aeruginosa* and *P. maltophilia*, now *Stenotrophomonas maltophilia*, are common isolates from the patient population I see. *P. cepacia*, now *Burkholderia cepacia* is also readily seen at Children's Medical Center because of our high cystic fibrosis population.

Accordingly, the ATCases from 16 members of the *Pseudomonas* genus and former *Pseudomonas* genus were studied in detail. The enzymes were partially purified and characterized for each species. These results are presented individually below.

*Pseudomonas aeruginosa* ATCase

*Pseudomonas aeruginosa* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 11-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (25-45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 4).
FIGURE 8. PAGE for Pseudomonas ATCases

Lane 1  E. coli
2  Pseudomonas aeruginosa
3  P. fluorescens
4  P. aureofaciens
5  P. putida
6  P. syringae
7  P. stutzeri
8  P. mendocina
9  P. pseudoalcaligenes
10  Burkholderia cepacia
Table 4. Purification of Aspartate Transcarbamoylase
from *Pseudomonas aeruginosa*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein µg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
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<td>58</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>52</td>
<td>19.8</td>
<td>1.0</td>
<td>52</td>
<td>83</td>
<td>96%</td>
<td>1.4</td>
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<tr>
<td>3</td>
<td>6</td>
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<td>10.7</td>
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<td>249</td>
<td>115%</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.7</td>
<td>1.6</td>
<td>8</td>
<td>629</td>
<td>15%</td>
<td>11</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
Heat treatment studies of the partially purified *P. aeruginosa* ATCase revealed that the enzyme retained 82% activity after heating for one minute at 60°C in ATCase buffer which decreased to 47% activity after heating for 10 minutes at 60°C. All activity was lost if the enzyme were heated for 30 minutes at 60°C while the enzyme retained 61% activity when heat treated for one minute at 70°C (Table 5). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. aeruginosa* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 9 and 10). A $K_m$ for aspartate of 1.0 mM and a $K_m$ for carbamoylphosphate of 1.6 mM were derived from Lineweaver-Burk plots (Figures 11 and 12).

The ATCase from *P. aeruginosa* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, ATP inhibited the activity of ATCase by 87%, UTP by 79%, and CTP by 71%. At saturating levels of aspartate (10 mM), enzyme activity was inhibited 85% by ATP, 77% by UTP, and only 16% by CTP (Figure 13). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was
TABLE 5. Specific Activity of Aspartate Transcarbamoylase
from *Pseudomonas aeruginosa* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
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<td>97</td>
<td></td>
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<td>0 Minutes</td>
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</tr>
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<td>1 Minutes</td>
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<td>46</td>
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<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>
FIGURE 9. Velocity-Substrate Curve for

*Pseudomonas aeruginosa*

![Velocity-Substrate Curve for Pseudomonas aeruginosa](image)

FIGURE 10. Velocity-Substrate Curve for

*Pseudomonas aeruginosa*

![Velocity-Substrate Curve for Pseudomonas aeruginosa](image)
FIGURE 11. Lineweaver-Burk Plot for

*Pseudomonas aeruginosa*

![Lineweaver-Burk Plot for Pseudomonas aeruginosa](image1)

\[ \frac{1}{v} \] vs. \[ \frac{1}{[\text{Aspartate}]} \]

- \[ K_m = 1.0 \text{ mM} \]

FIGURE 12. Lineweaver-Burk Plot for

*Pseudomonas aeruginosa*

![Lineweaver-Burk Plot for Pseudomonas aeruginosa](image2)

\[ \frac{1}{v} \] vs. \[ \frac{1}{[\text{Carbamoylphosphate}]} \]

- \[ K_m = 1.0 \text{ mM} \]
82% inhibition by ATP, 71% inhibition by UTP, and 30% inhibition by CTP (Figure 14).

\textit{Pseudomonas fluorescens} ATCase

\textit{Pseudomonas fluorescens} has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 39-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in \textit{Pseudomonas} minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 6).

Heat treatment studies of the partially purified \textit{P. fluorescens} ATCase revealed that enzyme was more enzymatically heating with heat for one minute at 60°C, 70°C, or 80°C. There was also increased ATCase activity when treated with heat for 10 minutes at 60°C and 90% retention of activity when heated at 60°C for 30 minutes (Table 7). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The \textit{P. fluorescens} ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations
FIGURE 13. Nucleotide Effector Response for

\[ Pseudomonas aeruginosa \]

![Graph showing nucleotide effector response for Pseudomonas aeruginosa.](image)

FIGURE 14. Nucleotide Effector Response for

\[ Pseudomonas aeruginosa \]

![Graph showing nucleotide effector response for Pseudomonas aeruginosa.](image)
Table 6. Purification of Aspartate Transcarbamoylase from *Pseudomonas fluorescens*

<table>
<thead>
<tr>
<th>Step</th>
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<th>Protein μg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37</td>
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<td>0.77</td>
<td>3.85</td>
<td>1421</td>
<td>6%</td>
<td>39</td>
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Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
<table>
<thead>
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<th>Time of Heating</th>
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<td>30 Minutes</td>
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</tbody>
</table>
(Figures 15 and 16). A $K_m$ for aspartate of 1.3 mM and a $K_m$ for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 17 and 18).

The ATCase from *P. fluorescens* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, UTP inhibited the activity of ATCase by 85%, ATP by 80%, and CTP by 71%. At saturating levels of aspartate (11 mM), enzyme activity was inhibited 90% by ATP, 82% by UTP, and 36% by CTP (Figure 19). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 96% inhibition by ATP, 81% inhibition by UTP, and 32% inhibition by CTP (Figure 20).

*Pseudomonas aureofaciens* ATCase

*Pseudomonas aureofaciens* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 60-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal
FIGURE 15. Velocity-Substrate Curve for *Pseudomonas fluorescens*

![Graph showing velocity-substrate curve for *Pseudomonas fluorescens* with substrate concentration on the x-axis and specific activity on the y-axis.]

FIGURE 16. Velocity-Substrate Curve for *Pseudomonas fluorescens*

![Graph showing velocity-substrate curve for *Pseudomonas fluorescens* with substrate concentration on the x-axis and specific activity on the y-axis.]

[Aspartate (mM)]

[Carbamoylphosphate (mM)]
FIGURE 17. Lineweaver-Burk Plot for

*Pseudomonas fluorescens*

\[ K_m = 1.3 \text{ mM} \]

1/velocity vs. 1/[Aspartate]

FIGURE 18. Lineweaver-Burk Plot for

*Pseudomonas fluorescens*

\[ K_m = 1.0 \text{ mM} \]

1/velocity vs. 1/[Carbamoylphosphate]
FIGURE 19. Nucleotide Effector Response for

*Pseudomonas fluorescens*

![Graph showing nucleotide effector response for *Pseudomonas fluorescens*. The x-axis represents [aspartate (mM)] with values at 1, 5, and 11. The y-axis represents specific activity in nmol CDA/min/mg protein. Different nucleotides (ATP, CTP, UTP) are shown with bars indicating their effect.]

FIGURE 20. Nucleotide Effector Response for

*Pseudomonas fluorescens*

![Graph showing nucleotide effector response for *Pseudomonas fluorescens*. The x-axis represents [carbamoylphosphate (mM)] with values at 0.16, 1.3, and 5.2. The y-axis represents specific activity in nmol CDA/min/mg protein. Different nucleotides (ATP, CTP, UTP) are shown with bars indicating their effect.]

medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 8).

Heat treatment studies of the partially purified *P. aureofaciens* ATCase revealed that there was a 40% loss in activity after heating at 60°C for up to 30 minutes. There was a loss of 60% ATCase activity, when the enzyme was heated for 10 minutes at 60°C, 70°C, or 80°C (Table 9). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. aureofaciens* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 21 and 22). A $K_m$ for aspartate of 1.3 mM and a $K_m$ for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 23 and 24).

The ATCase from *P. aureofaciens* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, neither CTP nor UTP inhibited the enzyme, but 44% inhibition of ATCase activity was seen with ATP. At saturating levels of aspartate (11 mM), no inhibition was seen with CTP, but activity was inhibited 76% by ATP and 70% by UTP (Figure 25). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of
Table 8. Purification of Aspartate Transcarbamoylase

from *Pseudomonas aureofaciens*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36</td>
<td>43.0</td>
<td>0.55</td>
<td>19.8</td>
<td>13</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>23.1</td>
<td>0.45</td>
<td>30.15</td>
<td>20</td>
<td>152%</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>22.8</td>
<td>1.8</td>
<td>10.8</td>
<td>75</td>
<td>55%</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.58</td>
<td>0.47</td>
<td>2.35</td>
<td>782</td>
<td>12%</td>
<td>60.2</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Minutes</td>
<td>47</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Minutes</td>
<td>29</td>
<td>38</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>10 Minutes</td>
<td>19</td>
<td>18</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>30 Minutes</td>
<td>35</td>
<td>12</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 21. Velocity-Substrate Curve for *Pseudomonas aureofaciens*

![Graph showing velocity-substrate curve for *Pseudomonas aureofaciens* with substrate concentration in mM on the x-axis and specific activity in nmol CAA/min/mg protein on the y-axis.]

FIGURE 22. Velocity-Substrate Curve for *Pseudomonas aureofaciens*

![Graph showing velocity-substrate curve for *Pseudomonas aureofaciens* with substrate concentration in mM on the x-axis and specific activity in nmol CAA/min/mg protein on the y-axis.]

FIGURE 23. Lineweaver-Burk Plot for Pseudomonas aureofaciens

\[ \frac{1}{V} = \frac{1}{V_{max}} \frac{1}{[S]} + \frac{1}{K_m} \]

- \( K_m = 1.3 \text{ mM} \)

FIGURE 24. Lineweaver-Burk Plot for Pseudomonas aureofaciens

\[ \frac{1}{V} = \frac{1}{V_{max}} \frac{1}{[S]} + \frac{1}{K_m} \]

- \( K_m = 1.0 \text{ mM} \)
carbamoylphosphate (5.2 mM), there was 100% inhibition by ATP and by UTP (Figure 26).

*Pseudomonas putida*

*Pseudomonas putida* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 60-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 10).

Heat treatment studies of the partially purified *P. putida* ATCase revealed that the enzyme was more enzymatically active after for heating 30 minutes at 60°C. Heating the enzyme for 10 minutes at 70°C still higher ATCase activity than the for control sample (Table 11). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. putida* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 27 and 28). A $K_m$ for aspartate of 1.0 mM and a $K_m$
FIGURE 25. Nucleotide Effector Response for *Pseudomonas aureofaciens*

![Bar graph showing the effect of nucleotides on specific activity in *Pseudomonas aureofaciens*.

**Y-axis:** Specific Activity (nmol CAA/min/mg protein)

**X-axis:** Aspartate (mM), values range from 0.5 to 11

- **No Effector**
- ATP
- CTP
- UTP

FIGURE 26. Nucleotide Effector Response for *Pseudomonas aureofaciens*

![Bar graph showing the effect of carbamoylphosphate on specific activity in *Pseudomonas aureofaciens*.

**Y-axis:** Specific Activity (nmol CAA/min/mg protein)

**X-axis:** Carbamoylphosphate (mM), values range from 0.16 to 5.2

- **No Effector**
- ATP
- CTP
- UTP
Table 10. Purification of Aspartate Transcarbamoylase
from *Pseudomonas putida*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein µg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>44.3</td>
<td>2.3</td>
<td>94.3</td>
<td>49</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>26.0</td>
<td>1.1</td>
<td>81.62</td>
<td>39</td>
<td>87%</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>35.3</td>
<td>10.6</td>
<td>63.5</td>
<td>278</td>
<td>67%</td>
<td>5.7</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.74</td>
<td>1.1</td>
<td>5.4</td>
<td>2923</td>
<td>6%</td>
<td>60</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction
<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Sample not Heated</th>
<th>Cell Extract 60°C</th>
<th>Cell Extract 70°C</th>
<th>Cell Extract 80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Minutes</td>
<td>63</td>
<td>97</td>
<td>73</td>
<td>69</td>
</tr>
<tr>
<td>1 Minute</td>
<td></td>
<td>97</td>
<td>79</td>
<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td></td>
<td>65</td>
<td>65</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 27. Velocity-Substrate Curve for *Pseudomonas putida*

FIGURE 28. Velocity-Substrate Curve for *Pseudomonas putida*
for carbamoylphosphate of 1.1 mM were derived from Lineweaver-Burk plots (Figures 29 and 30).

The ATCase from *P. putida* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, ATP inhibited the activity of ATCase by 95%, UTP by 79%, and CTP by 70%. At saturating levels of aspartate (9 mM), enzyme activity was inhibited 89% by ATP and 49% by UTP (Figure 31). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was still 93% inhibition by ATP, 62% inhibition by CTP, and 57% inhibition by UTP (Figure 32).

**Pseudomonas syringae** ATCase

*Pseudomonas syringae* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification. The enzyme was partially purified 6.5-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v)
FIGURE 29. Lineweaver-Burk Plot for 

*Pseudomonas putida*

\[ \frac{1}{V} \] vs \[ \frac{1}{[\text{Aspartate}]} \]

Km = 1.0 mM

FIGURE 30. Lineweaver-Burk Plot for 

*Pseudomonas putida*

\[ \frac{1}{V} \] vs \[ \frac{1}{[\text{Carbamoylphosphate}]} \]

Km = 1.1 mM
FIGURE 31. Nucleotide Effector Response for 

Pseudomonas putida

![Bar chart showing specific activity vs. aspartate concentration for different nucleotides.]

FIGURE 32. Nucleotide Effector Response for 

Pseudomonas putida

![Bar chart showing specific activity vs. carbamoylphosphate concentration for different nucleotides.]

---

[Graphical representation of the data showing the effects of different nucleotides on specific activity.]
Heat treatment studies of the partially purified *P. syringae* ATCase revealed that the enzyme retained 100% activity after heating for one minute at 60°C in ATCase buffer, which decreased to 64% activity after heating for 10 minutes at 60°C. All activity was lost if the enzyme were heated for 10 minutes at 70°C or above (Table 13). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. syringae* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 33 and 34). A $K_m$ for aspartate of 1.3 mM and a $K_m$ for carbamoylphosphate of 0.9 mM were derived from Lineweaver-Burk plots (Figures 35 and 36).

The ATCase from *P. syringae* was greatly inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. The enzyme had 100% inhibition by ATP, CTP, and UTP at low or saturating levels of aspartate or carbamoylphosphate (Figures 37 and 38).

*Pseudomonas stutzeri* ATCase

*Pseudomonas stutzeri* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8),
Table 12. Purification of Aspartate Transcarbamoylase from *Pseudomonas syringae*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>31.1</td>
<td>2.6</td>
<td>91.4</td>
<td>79</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>19.4</td>
<td>1.1</td>
<td>62.4</td>
<td>54</td>
<td>68%</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>27.6</td>
<td>7.1</td>
<td>42.8</td>
<td>213</td>
<td>47%</td>
<td>2.7</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1.2</td>
<td>6.2</td>
<td>11.2</td>
<td>508</td>
<td>12%</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
TABLE 13. Specific Activity of Aspartate Transcarbamoylase 
from *Pseudomonas syringae* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>77</td>
<td>16</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1 Minutes</td>
<td>28</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10 Minutes</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30 Minutes</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 33. Velocity-Substrate Curve for

*Pseudomonas syringae*

[Graph showing velocity-substrate curve for Pseudomonas syringae with substrate concentration on the x-axis and specific activity on the y-axis.]

FIGURE 34. Velocity-Substrate Curve for

*Pseudomonas syringae*

[Graph showing velocity-substrate curve for Pseudomonas syringae with substrate concentration on the x-axis and specific activity on the y-axis.]
FIGURE 35. Lineweaver-Burk Plot for

*Pseudomonas syringae*

\[ \frac{1}{V} \] vs. \[ \frac{1}{[\text{Aspartate}]} \]

\[ K_m = 1.3 \text{ mM} \]

FIGURE 36. Lineweaver-Burk Plot for

*Pseudomonas syringae*

\[ \frac{1}{V} \] vs. \[ \frac{1}{[\text{Carboxylylphosphate}]} \]

\[ K_m = 0.9 \text{ mM} \]
FIGURE 37. Nucleotide Effector Response for

Pseudomonas syringae

![Graph showing specific activity against different concentrations of aspartate and nucleotides.]

FIGURE 38. Nucleotide Effector Response for

Pseudomonas syringae

![Graph showing specific activity against different concentrations of carbamoylphosphate and nucleotides.]
nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 17-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 14).

Heat treatment studies of the partially purified *P. stutzeri* ATCase revealed that the enzyme retained 80% activity after heating for 30 minute at 60°C in ATCase buffer, which decreased to 50% activity after heating for one minutes at 80°C (Table 15). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. stutzeri* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 39 and 40). A $K_m$ for aspartate of 1.0 mM and a $K_m$ for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 41 and 42).

The ATCase from *P. stutzeri* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, ATP inhibited the activity of ATCase by 100%, UTP by 100%, and CTP by 61%. At saturating levels of aspartate (15 mM), the enzyme was inhibited 100% by ATP and
Table 14. Purification of Aspartate Transcarbamoylase
from *Pseudomonas stutzeri*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40</td>
<td>29.6</td>
<td>1.4</td>
<td>54.4</td>
<td>44</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>77</td>
<td>21.9</td>
<td>1.1</td>
<td>87.8</td>
<td>50</td>
<td>161%</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>21.4</td>
<td>11.8</td>
<td>82.5</td>
<td>511</td>
<td>150%</td>
<td>11.6</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.41</td>
<td>0.29</td>
<td>1.4</td>
<td>732</td>
<td>3%</td>
<td>17</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
TABLE 15. Specific Activity of Aspartate Transcarbamoylase
from Pseudomonas stutzeri Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>206</td>
<td>146</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>10 Minutes</td>
<td>159</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30 Minutes</td>
<td>159</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 39. Velocity-Substrate Curve for 
*Pseudomonas stutzeri*

![Graph](image)

FIGURE 40. Velocity-Substrate Curve for 
*Pseudomonas stutzeri*

![Graph](image)
FIGURE 41. Lineweaver-Burk Plot for *Pseudomonas stutzeri*

![Graph showing Lineweaver-Burk Plot for Pseudomonas stutzeri with K_m = 1.0 mM.]

FIGURE 42. Lineweaver-Burk Plot for *Pseudomonas stutzeri*

![Graph showing Lineweaver-Burk Plot for Pseudomonas stutzeri with k_m = 1.0 mM.]

UTP, and 25% by CTP (Figure 43). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 100% inhibition by ATP and UTP, and 25% inhibition seen by CTP (Figure 44).

*Pseudomonas mendocina* ATCase

*Pseudomonas mendocina* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 14.8-fold as described on Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 16).

Heat treatment studies of the partially purified *P. mendocina* ATCase revealed that the enzyme retained 70% activity after heating for one minute at 80°C in ATCase buffer, which decreased to 52% activity after heating for 10 minutes at 60°C (Table 17). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. mendocina* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted
FIGURE 43. Nucleotide effector response for *Pseudomonas stutzeri*

![Graph showing specific activity against aspartate concentration](image1)

FIGURE 44. Nucleotide effector response for *Pseudomonas stutzeri*

![Graph showing specific activity against carbamoylphosphate concentration](image2)
Table 16. Purification of Aspartate Transcarbamoylase
from Pseudomonas mendocina

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density (466 nm)</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34</td>
<td>35.8</td>
<td>1.3</td>
<td>45.6</td>
<td>36</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>26.2</td>
<td>0.9</td>
<td>53.7</td>
<td>33</td>
<td>118%</td>
<td>0.9</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>35.2</td>
<td>3.3</td>
<td>16.7</td>
<td>89</td>
<td>37%</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.56</td>
<td>0.12</td>
<td>0.85</td>
<td>534</td>
<td>2%</td>
<td>14.8</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
### TABLE 17. Specific Activity of Aspartate Transcarbamoylase from *Pseudomonas mendocina* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample not Heated</td>
<td>44</td>
<td>37</td>
<td>26</td>
<td>31</td>
</tr>
<tr>
<td>0 Minutes</td>
<td>37</td>
<td>26</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>1 Minutes</td>
<td>23</td>
<td>18</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10 Minutes</td>
<td>18</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
against either aspartate or carbamoylphosphate concentrations (Figures 45 and 46). A \( K_m \) for aspartate of 1.0 mM and a \( K_m \) for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 47 and 48).

The ATCase from *P. mendocina* was inhibited 100% by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM at low or saturating levels of aspartate or carbamoylphosphate (Figures 49 and 50).

*Pseudomonas pseudoalcaligenes* ATCase

*Pseudomonas pseudoalcaligenes* has a class A ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 8), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 8.3-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20–45% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 18).

Heat treatment studies of the partially purified *P. pseudoalcaligenes* ATCase revealed that the enzyme retained 92% activity after heating for one minute at 80°C in ATCase buffer. The enzyme remained 100% active after heating 30
FIGURE 45. Velocity-Substrate Curve for *Pseudomonas mendocina*

![Velocity-Substrate Curve](image)

FIGURE 46. Velocity-Substrate Curve for *Pseudomonas mendocina*

![Velocity-Substrate Curve](image)
FIGURE 47. Lineweaver-Burk Plot for *Pseudomonas mendocina*

![Lineweaver-Burk Plot for *Pseudomonas mendocina*](image1)

- $k_m = 1.0$ mM

FIGURE 48. Lineweaver-Burk Plot for *Pseudomonas mendocina*

![Lineweaver-Burk Plot for *Pseudomonas mendocina*](image2)

- $k_m = 1.0$ mM
FIGURE 49. Nucleotide Effector Response for Pseudomonas mendocina

FIGURE 50. Nucleotide Effector Response for Pseudomonas mendocina
Table 18. Purification of Aspartate Transcarbamoylase
from *Pseudomonas pseudoalcaligenes*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (μg/ml)</th>
<th>Optical Density (466 nm)</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41</td>
<td>54.4</td>
<td>1.95</td>
<td>80</td>
<td>34</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>35.3</td>
<td>0.9</td>
<td>66.9</td>
<td>24</td>
<td>84%</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>48.3</td>
<td>3.36</td>
<td>16.8</td>
<td>130</td>
<td>21%</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>1.07</td>
<td>0.296</td>
<td>2.1</td>
<td>282</td>
<td>2.6%</td>
<td>8.3</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 30 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
minutes at 60°C (Table 19). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. pseudoalcaligenes* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 51 and 52). A $K_m$ for aspartate of 1.0 mM and a $K_m$ for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 53 and 54).

The ATCase from *P. pseudoalcaligenes* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, ATP and UTP inhibited the activity of ATCase by 100% and CTP by 81%. At saturating levels of aspartate (9 mM), the enzyme activity was inhibited 100% by ATP or UTP, and 67% by CTP (Figure 55). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 100% inhibition seen by ATP, 100% inhibition seen by UTP, and 70% inhibition seen by CTP (Figure 56).

*Burkholderia cepacia* ATCase

*Burkholderia cepacia* has the largest ATCase seen thus far with a $M_r$ of approximately 600 kDa when observed on a gradient polyacrylamide gel (Figure 57). Uniquely, when the gradient polyacrylamide gel was electrophoresed at 4°C, a
TABLE 19. Specific Activity of Aspartate Transcarbamoylase from *Pseudomonas pseudoalcaligenes* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>29</td>
<td>23</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>1 Minutes</td>
<td>24</td>
<td>20</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10 Minutes</td>
<td>27</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>30 Minutes</td>
<td>27</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 51. Velocity-Substrate Curve for

*Pseudomonas pseudoalcaligenes*

[Graph showing specific activity vs. aspartate concentration]

FIGURE 52. Velocity-Substrate Curve for

*Pseudomonas pseudoalcaligenes*

[Graph showing specific activity vs. carbamoylphosphate concentration]
FIGURE 53. Lineweaver-Burk Plot for Pseudomonas pseudoalcaligenes

FIGURE 54. Lineweaver-Burk Plot for Pseudomonas pseudoalcaligenes
FIGURE 55. Nucleotide Effector Response for *Pseudomonas pseudoalcaligenes*

![Graph showing nucleotide effector response](image)

FIGURE 56. Nucleotide Effector Response for *Pseudomonas pseudoalcaligenes*

![Graph showing nucleotide effector response](image)
Figure 57. PAGE for Pseudomonad ATCases

<table>
<thead>
<tr>
<th>Lane</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>600 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>480 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>240 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 kDa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lane 1: *Pseudomonas aeruginosa*

2: *Burkholderia cepacia*

3: *B. pickettii*

4: *Comamonas acidovorans*

5: *C. testosteroni*

6: *Brevundimonas diminuta*

7: *Pseudomonas indigofera*

8: *Shewanella putrefaciens*

9: *Stenotrophomonas maltophilia*
smaller form of an active ATCase, with a $M_r$ of approximately 240 kDa, was observed (Figure 57).

The smaller ATCase was dissociated from the holoenzyme when passed over a Sephacryl 400S size-exclusion column or when passed over a HPLC anion exchange column during protein purification. Since all the steps of purification were carried out at 4°C, it was necessary to determine if the separation of the ATCase holoenzyme into a smaller form(s) was temperature-dependent. To that end, B. cepacia cell extract was treated with ammonium sulfate and passed over a Sephacryl 400S size-exclusion column at room temperature. Both forms of the ATCase from B. cepacia were present in different fractions when this procedure was carried out at room temperature. Gradient polyacrylamide gels were electrophoresed to confirm each form of ATCase from the individual fractions. The separation of the ATCases during the protein purification is seen in Figure 58.

Because the ATCase from B. cepacia represented an enzyme not previously observed, the ATCase holoenzyme was purified to homogeneity in order to determine the $M_r$ of the individual polypeptides. The ATCase holoenzyme (600 kDa) was first separated on a Sephacryl size-exclusion column. The fraction containing only this large ATCase form was then electrophoresed and electroeluted at 4°C to further remove impurities. The electroeluted ATCase was then passed over an
FIGURE 58. PAGE of ATCases from Burkholderia cepacia

Lane 1  Pseudomonas aeruginosa
2  P. aeruginosa
3  Burkholderia cepacia Cell Extract
4  B. cepacia Large ATCase from Sephacryl Column
5  B. cepacia Small ATCase from Sephacryl Column
6  B. cepacia Large ATCase from HPLC Anion Exchange Column
7  B. cepacia Small ATCase from HPLC Anion Exchange Column
8  Stenotrophomonas maltophilia
anion exchange column on the HPLC. Finally, SDS polyacrylamide gel electrophoresis was performed on the purified enzyme taken from the anion exchange column. As can be seen in Figure 59, the large ATCase of *B. cepacia* contained different polypeptides with molecular masses of 39 kDa and 60 kDa.

During the purification of the ATCase from *B. cepacia*, the 10% streptomycin sulfate step was omitted because it caused a 72% loss in enzyme activity. There was a 14.8-fold increase in ATCase activity after treatment with ammonium sulfate (25-40%) and passage over a Sephacryl size-exclusion column. There was a 129-fold increase after passage of the enzyme over an anion exchange column on the HPLC (Table 20).

Heat treatment of the holoenzyme (6 *pyrB* polypeptides and 6 *pyrC* polypeptides) of *B. cepacia* showed that the ATCase was 100% active after heating for 30 minutes to 60°C and after heating for one minute at 70°C (Table 21).

The holoenzyme of *Burkholderia cepacia*, defined being able to produce both 600 kDa and 240 kDa ATCase on non-denaturing polyacrylamide gel, exhibited sigmoidal saturation kinetics when the initial velocity was plotted against aspartate concentrations and Michaelis-Menten saturation kinetics when the initial velocity was plotted against carbamoylphosphat concentrations (Figures 60 and 61). A $K_m$ for aspartate of 5.0 mM and a $K_m$ for carbamoylphosphat of
FIGURE 59. SDS Gel of Purified ATcase from 
Burkholderia cepacia

Lane 1  Purified ATCase from Burkholderia cepacia
2  Pro-mega Mid-range Standard Marker
3  Partially Purified Small ATCase from B. cepacia
4  Partially Purified ATCase from B. cepacia
Table 20. Purification of Aspartate Transcarbamoylase from *Burkholderia cepacia*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein μg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>34 ml</td>
<td>45</td>
<td>1.14</td>
<td>38.8</td>
<td>12</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4.5 ml</td>
<td>39</td>
<td>3.1</td>
<td>14</td>
<td>37</td>
<td>36</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>6 ml</td>
<td>2.2</td>
<td>0.73</td>
<td>4.4</td>
<td>177</td>
<td>11</td>
<td>14.8</td>
</tr>
<tr>
<td>4</td>
<td>1.8 ml</td>
<td>0.045</td>
<td>0.11</td>
<td>0.2</td>
<td>1548</td>
<td>0.5</td>
<td>129</td>
</tr>
</tbody>
</table>

TABLE 21. Specific Activity of Aspartate Transcarbamoylase from *Burkholderia cepacia* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
<td></td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td></td>
<td>46</td>
<td>48</td>
<td>40</td>
</tr>
<tr>
<td>1 Minutes</td>
<td></td>
<td>49</td>
<td>41</td>
<td>13</td>
</tr>
<tr>
<td>10 Minutes</td>
<td></td>
<td>62</td>
<td>42</td>
<td>12</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 60. Velocity-Substrate Curve for Holoenzyme of *Burkholderia cepacia*

![Graph 60](image)

FIGURE 61. Velocity-Substrate Curve for Holoenzyme of *Burkholderia cepacia*

![Graph 61](image)
0.7 mM were derived from Lineweaver-Burk plots for the holoenzyme (Figures 62 and 63).

The "large" ATCase of *Burkholderia cepacia*, defined as having a slightly less 600 kDa form which does not dissociate into smaller forms on non-denaturing polyacrylamide gel. *Burkholderia cepacia* also exhibited sigmoidal saturation kinetics when the initial velocity was plotted against aspartate concentrations (Figures 64). A $K_m$ for aspartate of 4.0 mM was derived from Lineweaver-Burk plots for the large enzyme (Figure 66).

The small 240 kDa ATCase of *Burkholderia cepacia* exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against aspartate concentrations (Figures 65). A $K_m$ for aspartate of 1.0 mM was derived from Lineweaver-Burk plots for the small ATCase form (Figure 67).

The holoenzyme of *B. cepacia* was inhibited by ATP, CTP, or UTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (1.0 mM) and saturating carbamoylphosphate, the holoenzyme was inhibited 37% by UTP, 23% by ATP, and 22% by UTP (Figure 68). At saturating levels of aspartate (7 mM), the holoenzyme was inhibited 61% by ATP, 49% by CTP or UTP. At low carbamoylphosphate (0.33 mM), the holoenzyme was inhibited 49% by CTP, 43% by UTP, and 33% by ATP. At saturating levels of carbamoylphosphate (5.2 mM), the holoenzyme had 49% inhibition by ATP, 39% by CTP, and 30% by UTP (Figure 69).
FIGURE 62. Lineweaver-Burk Plot for Holoenzyme of *Burkholderia cepacia*

![Graph showing Lineweaver-Burk plot with a line and data points indicating $k_m = 5.0$ mM.]

FIGURE 63. Lineweaver-Burk Plot for Holoenzyme of *Burkholderia cepacia*

![Graph showing Lineweaver-Burk plot with a line and data points indicating $k_m = 0.7$ mM.]

$k_m$ indicates the Michaelis constant, which is a measure of the enzyme's affinity for its substrate.
FIGURE 64. Velocity-Substrate Curve for Large ATCase of *Burkholderia cepacia*

FIGURE 65. Velocity-Substrate Curve for Small ATCase of *Burkholderia cepacia*
FIGURE 66. Lineweaver-Burk Plot for Large ATCase of *Burkholderia cepacia*

![Graph for Large ATCase of *Burkholderia cepacia*](image)

**K_m** 4.0 mM

FIGURE 67. Lineweaver-Burk Plot for Small ATCase of *Burkholderia cepacia*

![Graph for Small ATCase of *Burkholderia cepacia*](image)

**K_m** 1.0 mM
FIGURE 68. Nucleotide Effector Response for Holoenzyme of *Burkholderia cepacia*

![Graph showing nucleotide effector response for holoenzyme of Burkholderia cepacia. The x-axis represents aspartate concentration in mM, ranging from 1 to 7. The y-axis represents specific activity in mmol CAA/min/mg protein, ranging from 0 to 2500. Different nucleotides (ATP, CTP, UTP) are indicated by different patterns. No Effector is also shown.]}

FIGURE 69. Nucleotide Effector Response for Holoenzyme of *Burkholderia cepacia*

![Graph showing nucleotide effector response for holoenzyme of Burkholderia cepacia. The x-axis represents carbamoylphosphate concentration in mM, ranging from 0.33 to 5.2. The y-axis represents specific activity in mmol CAA/min/mg protein, ranging from 0 to 2500. Different nucleotides (ATP, CTP, UTP) are indicated by different patterns. No Effector is also shown.]
At low aspartate concentration (1 mM) and saturating carbamoylphosphate, the "large" ATCase from *B. cepacia* was inhibited 49% by UTP, 29% by ATP, and 20% by UTP (Figure 70). At saturating levels of aspartate (7 mM), the "large" ATCase was inhibited 77% by ATP, 69% by CTP, and 41% by UTP. At low carbamoylphosphate (0.33 mM), the "large" ATCase was inhibited 83% by ATP, 73% by CTP, and 64% by UTP. At saturating levels of carbamoylphosphate (5.2 mM), the purified large was slightly inhibited by UTP or ATP, but was activated by CTP (Figure 71).

At low aspartate concentration (0.5 mM) and saturating carbamoylphosphate, the purified 240 kDa ATCase from *B. cepacia* saw massive activation by nucleotide effectors. At low aspartate concentration (0.5 mM), this small ATCase of *B. cepacia* had 98% activation by ATP and 67% activation by CTP (Figure 72). At low carbamoylphosphate concentrations (0.33 mM), the small ATCase had 124% activation by CTP, 112% activation by UTP, and 96% activation by ATP (Figure 73).

*Burkholderia pickettii* ATCase

*Burkholderia pickettii* has large ATCase with a molecular mass > 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 57), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 2.5-fold as described in Materials and Methods. Sonicated extracts, from cells
FIGURE 70. Nucleotide Effector Response for Large ATCase of Burkholderia cepacia

![Graph showing nucleotide effector response for Large ATCase of Burkholderia cepacia.](image1)

FIGURE 71. Nucleotide Effector Response for Large ATCase of Burkholderia cepacia

![Graph showing nucleotide effector response for Large ATCase of Burkholderia cepacia.](image2)
FIGURE 72. Nucleotide Effector Response for Small ATCase of *Burkholderia cepacia*.

- **Y-axis**: Specific Activity in nmol CAAs/min/mg protein
- **X-axis**: [Aspartate (mM)]
- **Legend**:
  - No Effector
  - ATP
  - CTP
  - UTP

FIGURE 73. Nucleotide Effector Response for Small ATCase of *Burkholderia cepacia*.

- **Y-axis**: Specific Activity in nmol CAAs/min/mg protein
- **X-axis**: [Carbamoylphosphate (mM)]
- **Legend**:
  - No Effector
  - ATP
  - CTP
  - UTP
grown to late-exponential phase in Pseudomonas minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-45% w/v); however, enzyme activity was lost when passed over a Sephacryl 400 S size-exclusion column (Table 22).

Heat treatment studies of the partially purified *B. pickettii* ATCase revealed the enzyme was extremely heat sensitive. After heating the enzyme for one minute at 60°C, there was a 49% loss in ATCase activity. Heating for longer times or increasing the temperature of the heating resulted in inactivation of the enzyme (Table 23). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *B. pickettii* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 74 and 75). A $K_m$ for aspartate of 3.5 mM and a $K_m$ for carbamoylphosphate of 0.9 mM were derived from Lineweaver-Burk plots (Figures 76 and 77).

The ATCase from *B. pickettii* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. The enzyme had 100% inhibition by ATP, CTP, and UTP at low or saturating levels of aspartate or carbamoylphosphate (Figures 78 and 79).

*Comamonas acidovorans* ATCase

*Comamonas acidovorans* has an ATCase with a molecular
Table 22. Purification of Aspartate Transcarbamoylase from Burkholderia pickettii

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein µg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>24.7</td>
<td>.35</td>
<td>8.8</td>
<td>15</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>47</td>
<td>18.0</td>
<td>.40</td>
<td>18.8</td>
<td>23</td>
<td>213%</td>
<td>1.5</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>16.5</td>
<td>.64</td>
<td>3.8</td>
<td>43</td>
<td>43%</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 25 to 45% Ammonium sulfate cut.
## TABLE 23. Specific Activity of Aspartate Transcarbamoylase

*from Burkholderia pickettii* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
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</tr>
<tr>
<td>0 Minutes</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE 74. Velocity-Substrate Curve for *Burkholderia pickettii*

![Graph showing the Velocity-Substrate Curve for *Burkholderia pickettii*.

**Y-axis:** Specific Activity (nmol CAFA/min/mg protein)

**X-axis:** [Aspartate (mM)]

Points on the graph represent experimental data, connected by a smooth curve.

FIGURE 75. Velocity-Substrate Curve for *Burkholderia pickettii*

![Graph showing the Velocity-Substrate Curve for *Burkholderia pickettii*.

**Y-axis:** Specific Activity (nmol CAFA/min/mg protein)

**X-axis:** [Carbamoylphosphate (mM)]

Points on the graph represent experimental data, connected by a smooth curve.
FIGURE 76. Lineweaver-Burk Plot for *Burkholderia pickettii*

![Lineweaver-Burk Plot for *Burkholderia pickettii*](image1)

Specific Activity

K\text{m} = 3.5 mM

FIGURE 77. Lineweaver-Burk Plot for *Burkholderia pickettii*

![Lineweaver-Burk Plot for *Burkholderia pickettii*](image2)

1/Velocity

K\text{m} = 0.9 mM
FIGURE 78. Nucleotide Effector Response for

![Graph showing nucleotide effector response for Burkholderia pickettii.]

FIGURE 79. Nucleotide Effector Response for

![Graph showing nucleotide effector response for Burkholderia pickettii.]

[Graph showing nucleotide effector response for Burkholderia pickettii.]

Specific Activity

nmol CAA/min/mg protein

[Aspartate (mM)]

0 5 10 15 20 25 30 35

[Carbamoylphosphate (mM)]

0.16 1.3 5.2

No effector
ATP
CTP
UTP

Burkholderia pickettii

No effector
ATP
CTP
UTP
mass > 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 57), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 35-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-50% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 24).

Heat treatment studies of the partially purified *C. acidovorans* ATCase revealed that the enzyme retained 100% activity after heating for 30 minutes at 60°C in ATCase buffer. However, temperatures above 60°C resulted in complete loss of enzyme activity (Table 25). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *C. acidovorans* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 80 and 81). A $K_m$ for aspartate of 0.6 mM and a $K_m$ for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 82 and 83).

The ATCase from *C. acidovorans* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1.0 mM. At low aspartate concentration and saturating
Table 24. Purification of Aspartate Transcarbamoylase
from Comamonas acidivorans

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density (466 nm)</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>34.5</td>
<td>2.97</td>
<td>130.7</td>
<td>81</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>20.6</td>
<td>1.2</td>
<td>96.0</td>
<td>56</td>
<td>52%</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>16.2</td>
<td>2.47</td>
<td>17.3</td>
<td>143</td>
<td>13%</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.251</td>
<td>0.74</td>
<td>3.7</td>
<td>2842</td>
<td>3%</td>
<td>35</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 20 to 50% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td></td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 Minutes</td>
<td></td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td></td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td>27</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE 80. Velocity-Substrate Curve for *Comamonas acidovorans*

![Graph showing the velocity-substrate curve for *Comamonas acidovorans* with specific activity as the y-axis and aspartate concentration as the x-axis.](image)

FIGURE 81. Velocity-Substrate Curve for *Comamonas acidovorans*

![Graph showing the velocity-substrate curve for *Comamonas acidovorans* with specific activity as the y-axis and carbamoylphosphate concentration as the x-axis.](image)
FIGURE 82. Lineweaver-Burk Plot for Comamonas acidovorans

Comamonas acidovorans

\[ K_m = 0.6 \text{ mM} \]

FIGURE 83. Lineweaver-Burk Plot for Comamonas acidovorans

Comamonas acidovorans

\[ K_m = 1.0 \text{ mM} \]
carbamoylphosphate, there was 100% inhibition by ATP, CTP, and UTP. At saturating aspartate concentrations, ATP and UTP inhibited the activity of ATCase by 100%, while CTP showed 73% inhibition (Figure 84). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 100% inhibition seen by ATP and UTP, and 91% inhibition seen by CTP (Figure 85).

Comamonas testosteroni ATCase

Comamonas testosteroni has an ATCase with a molecular mass > 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 57), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 18-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in Pseudomonas minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-50% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 26).

Heat treatment studies of the partially purified C. testosteroni ATCase revealed that the enzyme was extremely heat sensitive, losing 60% activity when heated for one minute at 60°C (Table 27). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.
FIGURE 84. Nucleotide Effector Response for Comamonas acidovorans

[Diagram showing specific activity in nmol CAA/min/mg protein as a response to various concentrations of aspartate (mM).]

FIGURE 85. Nucleotide Effector Response for Comamonas acidovorans

[Diagram showing specific activity in nmol CAA/min/mg protein as a response to various concentrations of carbamoylphosphate (mM).]
Table 26. Purification of Aspartate Transcarbamoylase
from Comamonas testosteroni

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein µg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>43.8</td>
<td>1.7</td>
<td>71.8</td>
<td>37</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>79</td>
<td>27.0</td>
<td>0.6</td>
<td>50.6</td>
<td>23</td>
<td>70</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>54.8</td>
<td>5.3</td>
<td>26.5</td>
<td>180</td>
<td>37</td>
<td>4.9</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>1.0</td>
<td>0.32</td>
<td>1.6</td>
<td>665</td>
<td>2</td>
<td>18.0</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 20 to 50% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
TABLE 27. Specific Activity of Aspartate Transcarbamoylase
from Comamonas testosteroni Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Cell Extract</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not Heated</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
The *C. testosteroni* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 86 and 87). A $K_m$ for aspartate of 1.0 mM and a $K_m$ for carbamoylphosphate of 0.7 mM were derived from Lineweaver-Burk plots (Figures 88 and 89).

The ATCase from *C. testosteroni* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. The enzyme had 100% inhibition by ATP, CTP, and UTP at low or saturating levels of aspartate or carbamoylphosphate (Figures 90 and 91).

*Brevundimonas diminuta* ATCase

*Brevundimonas diminuta* has an ATCase with a molecular mass of approximately 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 57), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 6.4-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-55% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 28).

Heat treatment studies of the partially purified *B. diminuta* ATCase revealed that the enzyme retained 100% activity after heating for 30 minutes at 60°C in ATCase
FIGURE 86. Velocity-Substrate Curve for Comamonas testosteroni

FIGURE 87. Velocity-Substrate Curve for Comamonas testosteroni
FIGURE 88. Lineweaver-Burk Plot for *Comamonas testosteroni*

![Lineweaver-Burk Plot for *Comamonas testosteroni*](image)

$K_m = 1.0 \text{ mM}$

FIGURE 89. Lineweaver-Burk Plot for *Comamonas testosteroni*

![Lineweaver-Burk Plot for *Comamonas testosteroni*](image)

$K_m = 0.7 \text{ mM}$
FIGURE 90. Nucleotide Effector Response for

Comamonas testosteroni

![Graph showing nucleotide effector response for Comamonas testosteroni. The x-axis represents Aspartate (mM) with values 0.25, 3, and 7. The y-axis represents Specific Activity in mmol CAAM/min/mg protein, ranging from 0 to 600. Bars indicate no effector and the presence of ATP, CTP, and UTP at different concentrations.]

FIGURE 91. Nucleotide Effector Response for

Comamonas testosteroni

![Graph showing nucleotide effector response for Comamonas testosteroni. The x-axis represents Carbamoylphosphate (mM) with values 0.16, 1.3, and 5.2. The y-axis represents Specific Activity in mmol CAAM/min/mg protein, ranging from 0 to 600. Bars indicate no effector and the presence of ATP, CTP, and UTP at different concentrations.]

[The figures depict the effect of different nucleotides on the specific activity of an enzyme in Comamonas testosteroni under varying concentrations of aspartate and carbamoylphosphate.]
Table 28. Purification of Aspartate Transcarbamoylase from Brevundimonas diminuta

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume ml</th>
<th>Protein µg/ml</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39</td>
<td>10.28</td>
<td>0.38</td>
<td>14.6</td>
<td>37</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>25.8</td>
<td>0.27</td>
<td>17.8</td>
<td>14</td>
<td>121%</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>14.0</td>
<td>0.62</td>
<td>3.1</td>
<td>44</td>
<td>21%</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.46</td>
<td>0.08</td>
<td>0.38</td>
<td>236</td>
<td>3%</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 20 to 55% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
buffer. (Table 29). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *B. diminuta* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 92 and 93). A $K_m$ for aspartate of 1.0 mM and a $K_m$ for carbamoylphosphate of 0.9 mM were derived from Lineweaver-Burk plots (Figures 94 and 95).

The ATCase from *B. diminuta* had no inhibition by ATP, CTP, or UTP at low aspartate concentrations and carbamoylphosphate saturation. At saturation concentrations of aspartate, there was no inhibition by ATP or UTP and slight activation in ATCase activity by CTP (Figure 96). At low levels of carbamoylphosphate (0.33 mM), there was 100% inhibition by ATP, CTP, and UTP, whereas, at saturating levels of carbamoylphosphate, there was only 38% inhibition seen by UTP and no inhibition seen with either ATP or CTP (Figure 97).

*Pseudomonas indigofera* ATCase

*Pseudomonas indigofera* has an ATCase with a molecular mass < 480 kDa. It does not dissociate into subunits on a non-denaturing gradient polyacrylamide gel when electrophoresed at 4°C (Figure 57), nor were any active dissociated subunits observed during protein purification.

The enzyme was partially purified 30-fold as described in Materials and Methods. Sonicated extracts, from cells
TABLE 29. Specific Activity of Aspartate Transcarbamoylase 

from Brevundimonas diminuta Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Control</th>
<th>50°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not heated</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>15</td>
<td>16</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>1 Minutes</td>
<td>16</td>
<td>9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10 Minutes</td>
<td>16</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE 92. Velocity-Substrate Curve for *Brevundimonas diminuta*

![Velocity-Substrate Curve for *Brevundimonas diminuta*](image)

Specific Activity
nmol CAA/min/mg protein

[Aspartate (mM)]

FIGURE 93. Velocity-Substrate Curve for *Brevundimonas diminuta*

![Velocity-Substrate Curve for *Brevundimonas diminuta*](image)

Specific Activity
nmol CAA/min/mg protein

[Carbamoylphosphate (mM)]
FIGURE 94. Lineweaver-Burk Plot for *Brevundimonas diminuta*

$K_m = 1.0 \text{ mM}$

FIGURE 95. Lineweaver-Burk Plot for *Brevundimonas diminuta*

$K_m = 0.9 \text{ mM}$
FIGURE 96. Nucleotide Effector Response for

*Brevundimonas diminuta*

![Graph showing nucleotide effector response for Brevundimonas diminuta. The x-axis represents [Aspartate (mM)] with values at 0.25, 1, and 5. The y-axis represents specific activity in nmol CAU/min/mg protein. The graph shows different bars for No Effector, ATP, CTP, and UTP, with ATP showing the highest activity at 5 mM aspartate.]

FIGURE 97. Nucleotide Effector Response for

*Brevundimonas diminuta*

![Graph showing nucleotide effector response for Brevundimonas diminuta. The x-axis represents [Carbamoylphosphate (mM)] with values at 0.33, 2.5, and 10.4. The y-axis represents specific activity in nmol CAU/min/mg protein. The graph shows different bars for No Effector, ATP, CTP, and UTP, with ATP showing the highest activity at 10.4 mM carbamoylphosphate.]

grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (25-45% w/v) and size-exclusion chromatography on a Sephacryl 400S column (Table 30).

Heat treatment studies of the partially purified *P. indigofera* ATCase revealed that the enzyme retained 100% activity after heating 10 minutes at 60°C in ATCase buffer. However, temperatures above 60°C resulted in loss of enzyme activity (Table 31). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *P. indigofera* ATCase exhibited sigmoidal saturation kinetics when the initial velocity was plotted against aspartate concentration (Figure 98). The *P. indigofera* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against carbamoylphosphate concentration (Figure 99). A $K_m$ for aspartate of 9.0 mM and a $K_m$ for carbamoylphosphate of 0.7 mM were derived from Lineweaver-Burk plots (Figures 100 and 101).

The ATCase from *P. indigofera* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration (3.0 mM) and saturating carbamoylphosphate, CTP inhibited the activity of ATCase by 49% and ATP by 37%. At saturating levels of aspartate (15 mM), enzyme activity was slightly activated by CTP and UTP (Figure 102). At low carbamoylphosphate concentration (0.16
Table 30. Purification of Aspartate Transcarbamoylase
from *Pseudomonas indigofera*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity nmol/min/mg protein</th>
<th>Yield %</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>17.8</td>
<td>5.2</td>
<td>130</td>
<td>272</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>33.5</td>
<td>2.5</td>
<td>125</td>
<td>70</td>
<td>96%</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>30.8</td>
<td>18.2</td>
<td>109</td>
<td>547</td>
<td>84%</td>
<td>2.0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>.46</td>
<td>.935</td>
<td>23.4</td>
<td>2020</td>
<td>18%</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 25 to 45% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
TABLE 31. Specific Activity of Aspartate Transcarbamoylase from *Pseudomonas indigofera* Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Control</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not heated</td>
<td></td>
<td>46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td></td>
<td>49</td>
<td>33</td>
<td>24</td>
</tr>
<tr>
<td>1 Minutes</td>
<td></td>
<td>56</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td></td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE 98. Velocity-Substrate Curve for

*Pseudomonas indigofera*

![Graph showing specific activity vs. [Aspartate (mM)] for *Pseudomonas indigofera*.

FIGURE 99. Velocity-Substrate Curve for

*Pseudomonas indigofera*

![Graph showing specific activity vs. [Carbamoylphosphate (mM)] for *Pseudomonas indigofera*.
FIGURE 100. Lineweaver-Burk Plot for 

*Pseudomonas indigofera*

![Graph showing Lineweaver-Burk Plot for Pseudomonas indigofera with a 
$K_m$ of 9.0 mM.]

FIGURE 101. Lineweaver-Burk Plot for 

*Pseudomonas indigofera*

![Graph showing Lineweaver-Burk Plot for Pseudomonas indigofera with a 
$K_m$ of 0.7 mM.]

$K_m$ refers to the Michaelis constant, which is the substrate concentration at which the reaction rate is half of its maximum.
mM), there was 100% inhibition by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 36% inhibition seen by CTP (Figure 103).

Shewanella putrefaciens ATCase

Shewanella putrefaciens had an aspartate transcarbamoylase with a molecular mass of 100 kDa (Figure 57).

The enzyme was partially purified 101-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in Pseudomonas minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-60% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 32).

Heat treatment studies of the partially purified S. putrefaciens ATCase revealed that the enzyme retained 100% activity after heating for one minute at 60°C in ATCase buffer. However, temperatures above 60°C resulted in loss of enzyme activity (Table 33). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The S. putrefaciens ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted against either aspartate or carbamoylphosphate concentrations (Figures 104 and 105). A $K_m$ for aspartate of 0.6 mM and a $K_m$ for carbamoylphosphate of 1.0 mM were derived from Lineweaver-Burk plots (Figures 106 and 107).
FIGURE 102. Nucleotide Effector Response for

*Pseudomonas indigofera*

![Graph showing nucleotide effector response for Pseudomonas indigofera](image)

FIGURE 103. Nucleotide Effector Response for

*Pseudomonas indigofera*

![Graph showing nucleotide effector response for Pseudomonas indigofera](image)
Table 32. Purification of Aspartate Transcarbamoylase

from *Shewanella putrefaciens*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (µg/ml)</th>
<th>Optical Density 466 nm</th>
<th>Total (Units)</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield (%)</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>11.2</td>
<td>0.6</td>
<td>15</td>
<td>53</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>15.0</td>
<td>0.38</td>
<td>19</td>
<td>26</td>
<td>126%</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>11.9</td>
<td>8.43</td>
<td>13</td>
<td>658</td>
<td>34%</td>
<td>12.5</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.05</td>
<td>0.35</td>
<td>51</td>
<td>5330</td>
<td>14%</td>
<td>101</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 20 to 60% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
TABLE 33. Specific Activity of Aspartate Transcarbamoylase
from Shewanella putrefaciens Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Control</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not heated</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 Minutes</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
FIGURE 104. Velocity-Substrate Curve for

*Shewanella putrefaciens*

![Graph showing specific activity vs. aspartate concentration](image1)

FIGURE 105. Velocity-Substrate Curve for

*Shewanella putrefaciens*

![Graph showing specific activity vs. carbamoylphosphate concentration](image2)
FIGURE 106. Lineweaver-Burk Plot for *Shewanella putrefaciens*

![Lineweaver-Burk Plot for *Shewanella putrefaciens*](image1)

$K_m = 2.0 \text{ mM}$

FIGURE 107. Lineweaver-Burk Plot for *Shewanella putrefaciens*

![Lineweaver-Burk Plot for *Shewanella putrefaciens*](image2)

$K_m = 0.5 \text{ mM}$
The ATCase from *S. putrefaciens* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At saturating aspartate concentrations, the enzyme was inhibited 24% by ATP and 23% by CTP (Figure 108). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 27% inhibition seen by ATP and 23% inhibition seen by CTP (Figure 109).

*Stenotrophomonas maltophilia* ATCase

*Stenotrophomonas maltophilia* had an ATCase with a molecular mass of 100 kDa (Figure 57).

The enzyme was partially purified 8-fold as described in Materials and Methods. Sonicated extracts, from cells grown to late-exponential phase in *Pseudomonas* minimal medium, were treated successively with streptomycin sulfate (10% w/v), ammonium sulfate (20-55% w/v) and size-exclusion chromatography on a Sephacryl 400 S column (Table 34).

Heat treatment studies of the partially purified *S. maltophilia* ATCase revealed that the enzyme retained 100% activity after heating for 30 minutes at 60°C in ATCase buffer. The enzyme retained full activity when heated one for minute at 80°C (Table 35). The partially purified ATCase was stable for at least one month at 4°C in ATCase buffer.

The *S. maltophilia* ATCase exhibited Michaelis-Menten saturation kinetics when the initial velocity was plotted
FIGURE 108. Nucleotide Effector Response for *Shewanella putrefaciens*

![Graph showing specific activity of *Shewanella putrefaciens* with varying aspartate concentrations. The y-axis represents specific activity in nmol CA/min/mg protein, and the x-axis represents [aspartate (mM)].](image1)

FIGURE 109. Nucleotide Effector Response for *Shewanella putrefaciens*

![Graph showing specific activity of *Shewanella putrefaciens* with varying carbamoylphosphate concentrations. The y-axis represents specific activity in nmol CA/min/mg protein, and the x-axis represents [carbamoylphosphate (mM)].](image2)
Table 34. Purification of Aspartate Transcarbamoylase from *Stenotrophomonas maltophilia*

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (μg/ml)</th>
<th>Optical Density 466 nm</th>
<th>Total Units</th>
<th>Specific Activity (nmol/min/mg protein)</th>
<th>Yield</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>12.5</td>
<td>0.56</td>
<td>14</td>
<td>44</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>11.2</td>
<td>0.41</td>
<td>20.5</td>
<td>37</td>
<td>146%</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>14.8</td>
<td>0.85</td>
<td>5.1</td>
<td>56</td>
<td>36%</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>0.46</td>
<td>0.14</td>
<td>0.8</td>
<td>359</td>
<td>6%</td>
<td>8</td>
</tr>
</tbody>
</table>

Step 1. Cell extract, 2. 10% Streptomycin sulfate cut, 3. 20 to 55% Ammonium sulfate cut, 4. Sephacryl 400S fraction.
TABLE 35. Specific Activity of Aspartate Transcarbamoylase
from Stenotrophomonas maltophilia Treated with Heat

<table>
<thead>
<tr>
<th>Time of Heating</th>
<th>Control</th>
<th>60°C</th>
<th>70°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample not heated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 Minutes</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Minutes</td>
<td></td>
<td>25</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>10 Minutes</td>
<td></td>
<td>34</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 Minutes</td>
<td></td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
against either aspartate or carbamoylphosphate concentrations (Figures 110 and 111). A $K_m$ for aspartate of 0.7 mM and a $K_m$ for carbamoylphosphate of 0.7 mM were derived from Lineweaver-Burk plots (Figures 112 and 113).

The ATCase from *S. maltophilia* was inhibited by ATP, UTP, and CTP when the effector concentration was maintained at 1 mM. At low aspartate concentration and saturating carbamoylphosphate, there was 56% inhibition by ATP, 54% by CTP, and 40% by UTP. At saturating aspartate concentrations, the enzyme was inhibited 42% by CTP and 30% by ATP (Figure 114). At low carbamoylphosphate concentration (0.16 mM), there was 100% inhibition of ATCase activity by ATP, CTP, or UTP, while at saturating levels of carbamoylphosphate (5.2 mM), there was 45% inhibition seen by CTP, 40% by ATP, and only 27% inhibition seen by UTP (Figure 115).
FIGURE 110. Velocity-Substrate Curve for *Stenotrophomonas maltophilia*

![Velocity-Substrate Curve for Stenotrophomonas maltophilia](image110)

FIGURE 111. Velocity-Substrate Curve for *Stenotrophomonas maltophilia*

![Velocity-Substrate Curve for Stenotrophomonas maltophilia](image111)
FIGURE 112. Lineweaver-Burk Plot for *Stenotrophomonas maltophilia*:

![Graph showing Lineweaver-Burk Plot with a slope line and points indicating an approximate $K_m$ of 0.7 mM.]

FIGURE 113. Lineweaver-Burk Plot for *Stenotrophomonas maltophilia*:

![Graph showing Lineweaver-Burk Plot with a slope line and points indicating an approximate $K_m$ of 0.7 mM.]

174a
FIGURE 114. Nucleotide Effector Response for *Stenotrophomonas maltophilia*

![Graph showing nucleotide effector response for Stenotrophomonas maltophilia](image)

FIGURE 115. Nucleotide Effector Response for *Stenotrophomonas maltophilia*

![Graph showing nucleotide effector response for Stenotrophomonas maltophilia](image)
Classification within the genus *Pseudomonas* is in a state of flux at the present time. When this study was initiated, reorganization of the genus had already begun. The results of this study, using aspartate transcarbamoylase (ATCase) as a taxonomic marker, are in agreement with the assignment of *Pseudomonas* species to new genera. During the course of this study, not only were differences seen for ATCases in different organisms that suggested that the organisms were not related, but there were ATCases observed that had not been observed or characterized previously. Indeed, a whole new class of ATCase was discovered.

*Pseudomonas aeruginosa*, *P. fluorescens*, *P. aureofaciens*, *P. putida*, *P. syringae*, *P. stutzeri*, *P. mendocina*, and *P. pseudoalcaligenes* remain as the genus *Pseudomonas sensu stricto*, which were part of the rRNA homology group I. All these pseudomonads have ATCases with $M_r$ of approximately 480 kDa, which could not be dissociated into smaller forms than the 480 kDa with any procedure during this study. All exhibited Michaelis–Menten kinetics when aspartate or
carbamoylphosphate velocity-substrate curves were plotted. The $K_m$ values obtained were very similar for all members of this group ranging from 1.0 to 1.3 mM when the aspartate concentrations were varied and $K_M$ values of 0.9 to 1.1 mM when the carbamoylphosphate concentrations were varied. The *Pseudomonas* ATCases are compared in Table 36.

The ATCases from all eight true pseudomonads gave maximal inhibition with ATP greater than UTP greater than CTP whether aspartate or carbamoylphosphate was varied. The enzymes from *P. syringae* and *P. mendocina* were inhibited 100 per cent under all conditions measured, while all eight ATCases had 100 per cent inhibition by nucleotide effectors at low carbamoylphosphate concentrations. Nucleotide effector responses are compared in Tables 37 and 38. Thus, the results obtained in this study validate the earlier finding that the ATCases of the eight rRNA homology group I members are representative of class A ATCases.

Members of the former rRNA homology group II, which included *Burkholderia cepacia* and *B. pickettii*, have been found to contain much larger ATCases than seen previously. The ATCase of *B. cepacia* has a $M_r$ of approximately 600 kDa and most surprisingly, contained a fully active dissociated form of ATCase of $M_r$ of approximately 240 kDa never seen previously (Table 36). This smaller ATCase, which dissociated from the 600 kDa holoenzyme, was first observed on polyacrylamide gel electrophoresis. This dissociated
<table>
<thead>
<tr>
<th>Organism</th>
<th>Mr (kDa)</th>
<th>Dissociates</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; ASP (nmol/min/mg protein)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; ASP (mM)</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; CP (nmol/min/mg protein)</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; CP (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>-480</td>
<td>No</td>
<td>625</td>
<td>1.0</td>
<td>625</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>-480</td>
<td>No</td>
<td>1225</td>
<td>1.3</td>
<td>1203</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td>-480</td>
<td>No</td>
<td>700</td>
<td>1.3</td>
<td>680</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>-480</td>
<td>No</td>
<td>3000</td>
<td>1.0</td>
<td>2803</td>
<td>1.1</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>-480</td>
<td>No</td>
<td>755</td>
<td>1.3</td>
<td>754</td>
<td>0.9</td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td>-480</td>
<td>No</td>
<td>818</td>
<td>1.0</td>
<td>775</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. mendocina</em></td>
<td>-480</td>
<td>No</td>
<td>632</td>
<td>1.0</td>
<td>553</td>
<td>1.0</td>
</tr>
<tr>
<td><em>P. pseudoalcaligenes</em></td>
<td>-480</td>
<td>No</td>
<td>913</td>
<td>1.0</td>
<td>800</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>-600</td>
<td>Yes</td>
<td>2550</td>
<td>5.0</td>
<td>1825</td>
<td>0.7</td>
</tr>
<tr>
<td><em>B. pickettii</em></td>
<td>-500</td>
<td>No</td>
<td>30</td>
<td>3.5</td>
<td>60</td>
<td>0.9</td>
</tr>
<tr>
<td><em>Comamonas acidovorans</em></td>
<td>-500</td>
<td>No</td>
<td>3776</td>
<td>0.6</td>
<td>3100</td>
<td>1.0</td>
</tr>
<tr>
<td><em>C. testosteroni</em></td>
<td>-500</td>
<td>No</td>
<td>570</td>
<td>1.0</td>
<td>410</td>
<td>0.7</td>
</tr>
<tr>
<td><em>Brevundimonas diminuta</em></td>
<td>-480</td>
<td>No</td>
<td>139</td>
<td>1.0</td>
<td>104</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Pseudomonas indigofera</em></td>
<td>-400</td>
<td>No</td>
<td>1714</td>
<td>9.0</td>
<td>1070</td>
<td>0.7</td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em></td>
<td>-100</td>
<td>No</td>
<td>6536</td>
<td>2.0</td>
<td>5400</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>-100</td>
<td>No</td>
<td>750</td>
<td>0.7</td>
<td>768</td>
<td>0.7</td>
</tr>
</tbody>
</table>
TABLE 37. Comparison of Nucleotide Effectors Response on ATCase by Varying Aspartate Concentrations

<table>
<thead>
<tr>
<th>Organism</th>
<th>Limiting [Asp] % Inhibition</th>
<th>Saturating [Asp] % Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP</td>
<td>CTP</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>87</td>
<td>71</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>80</td>
<td>71</td>
</tr>
<tr>
<td><em>P. aureofaciens</em></td>
<td>74</td>
<td>62</td>
</tr>
<tr>
<td><em>P. putida</em></td>
<td>95</td>
<td>70</td>
</tr>
<tr>
<td><em>P. syringae</em></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>P. stutzeri</em></td>
<td>100</td>
<td>61</td>
</tr>
<tr>
<td><em>P. mendocina</em></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>P. pseudoalcaligenes</em></td>
<td>100</td>
<td>81</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holoenzyme</td>
<td>23</td>
<td>37</td>
</tr>
<tr>
<td>Large ATCase</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>Small ATCase</td>
<td>A*</td>
<td>23</td>
</tr>
<tr>
<td><em>B. pickettii</em></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Comamonas acidovorans</em></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>C. testosteroni</em></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Brevundimonas diminuta</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Pseudomonas indigofera</em></td>
<td>73</td>
<td>49</td>
</tr>
<tr>
<td><em>Shewanella putrefaciens</em></td>
<td>56</td>
<td>54</td>
</tr>
<tr>
<td><em>Stenotrophomonas</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>maltophilia</em></td>
<td>56</td>
<td>54</td>
</tr>
</tbody>
</table>

A* = Activation of ATCase Activity
TABLE 38. Comparison of Nucleotide Effectors Response on ATCase by Varying Carbamoylphosphate Concentrations

<table>
<thead>
<tr>
<th>Organism</th>
<th>Limiting [CP]</th>
<th>Saturating [CP]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>CTP</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. aureofaciens</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. putida</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. syringae</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. stutzeri</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. mendocina</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P. pseudoalcaligenes</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Holoenzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>49</td>
</tr>
<tr>
<td>Large ATCase</td>
<td>83</td>
<td>73</td>
</tr>
<tr>
<td>Small ATCase</td>
<td>A*</td>
<td>A*</td>
</tr>
<tr>
<td>B. picketti</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Comamonas acidivorans</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C. testosteroni</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brevundimonas diminuta</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas indigofera</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Shewanella putrefaciens</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

A* = Activation of ATCase Activity
ATCase was also seen when B. cepacia extracts were passed over a Sephacryl 400S size-exclusion column or over an anion exchange column. A proposed model for the B. cepacia ATCase is presented in Figure 116. For the purpose of discussion, the holoenzyme, henceforth defined as the 600 kDa dodecamer dissociated into 600 kDa and 240 kDa forms (Figure 58, Lane 3). The "large ATCase" is defined as a dodecamer minus one or two of the larger polypeptides that does not dissociate further (Figure 58, Lane 6). These three ATCase forms are depicted in Figure 117. Results obtained from SDS analysis showed that the enzyme from B. cepacia contained two polypeptides with $M_r$ of 39 kDa and 60 kDa. The 39 kDa polypeptide size has not been seen in any previously reported ATCases, as all known ATCases contain a smaller 34 kDa polypeptide.

Both holoenzyme and "large" ATCase from B. cepacia had sigmoidal curves when aspartate concentration was varied. Both had similar [S]$_{0.5}$ values of 5.0 mM and 4.0 mM respectively. The small 240 kDa ATCase exhibited Michaelis-Menten kinetics when aspartate concentration was varied and had a $K_M$ value of 1.0 mM. One can imagine that the small 240 kDa ATCase of B. cepacia behaves like the dissociated catalytic trimer of E. coli, but the unlike the E. coli trimer, the small form of ATCase of B. cepacia was much less active than the holoenzyme of B. cepacia. ATCase characteristics of B. cepacia are shown in Table 39.
FIGURE 116. Model for *Burkholderia cepacia* ATCase

- **60 kDa Polypeptide** (pyrC')
- **39 kDa Polypeptide** (pyrB)
- One Catalytic polypeptide of trimer
- Trimer + 2
  - $M_r \sim 237$ kDa
- Trimer + 1
  - $M_r \sim 177$ kDa
- Trimer Only
  - Not Active
- Trimer + 3
  - $M_r \sim 297$ kDa
FIGURE 117. Suggested forms of *Burkholderia cepacia* ATCases

**Holoenzyme ~ 600 kDa**

- 60 kDa
- One catalytic polypeptide of trimer
- 39 kDa

**Large ATCase ~ 540 kDa**

**Small ATCase ~ 240 kDa**
TABLE 39. Comparison of ATCases from *Burkholderia cepacia*

<table>
<thead>
<tr>
<th>ATCase</th>
<th>Curve</th>
<th>$V_{max}$ [Asp]</th>
<th>$[S]_{0.5}$ [Asp]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holoenzyme</td>
<td>Sigmoidal</td>
<td>2505</td>
<td>5.0 mM</td>
</tr>
<tr>
<td>Large ATCase</td>
<td>Sigmoidal</td>
<td>900</td>
<td>4.0 mM</td>
</tr>
<tr>
<td>Small ATCase</td>
<td>Hyperbolic</td>
<td>415</td>
<td>1.0 mM</td>
</tr>
</tbody>
</table>

All three forms of ATCases of *B. cepacia* displayed hyperbolic curves when carbamoylphosphate was the limiting substrate and all three had similar $K_M$ values of 1.0 mM for carbamoylphosphate.

Differences in nucleotide effector response were seen between the holoenzyme, the "large" ATCase, and the small 240 kDa ATCase of *B. cepacia* (Tables 37 and 38). The most striking result was the activation of ATCase activity when nucleotide effector response was measured for the small ATCase. At low aspartate concentration (0.5 mM), the 240 kDa ATCase showed 98 per cent activation by ATP and 67 per cent by CTP. Similarly, at low carbamoylphosphate concentration (0.33 mM) the small ATCase was 96 per cent activated by ATP, 124 per cent by CTP, and 112 per cent by UTP.

Although *Burkholderia pickettii* was placed in the same genus as *B. cepacia*, the *B. pickettii* behaved differently than did *B. cepacia*. The ATCase holoenzyme from this organism was slightly smaller with a $M_r$ of approximately 500
kDa and never dissociated into active smaller molecular weight forms. There were also differences seen during the purification process. The *B. cepacia* enzyme was quite stable during purification, whereas, *B. pickettii* ATCase was completely inactivated when passed over a Sephacryl size-exclusion column. Ammonium sulfate treated cell extracts from *B. pickettii* retained ATCase activity when passed over an anion exchange column. The ATCase of *B. pickettii* exhibited Michaelis-Menten kinetics under all conditions of assay, whereas, a sigmoidal saturation curve was seen when aspartate was varied for *B. cepacia*.

The enzyme from *B. pickettii* gave a different nucleotide effector response (Table 37 and 38) relative to *B. cepacia*, showing 100 per cent inhibition by ATP, CTP, or UTP when either the concentration of aspartate or carbamoylphosphate were varied.

It is suggested that the ATCases discovered here seen in the genus *Burkholderia* represent a new class of ATCase not previously reported. This is certainly true for *B. cepacia* if not for *B. pickettii*. At the present time, there are three classes of bacterial ATCase classes, but the ATCases of this genus do not fit into any of these classes. Thus, I propose a new class of ATCases to accommodate the larger, dissociating bacterial ATCases. It is necessary to wait on genetic studies before such a class is named. Then it will be possible to decide between a subset of the present class A
(class A1) and a completely new class (class D). The DNA work is underway in our laboratory.

The unique nature of the small dissociated subunits of B. cepacia suggests that it may be useful as a probe for the quick identification of this organism. Because the organism is a potentially lethal pathogen in persons having cystic fibrosis (CF), which can readily be passed from one person to another person having CF, a quick way to probe for the organism directly from the patients sputum would be very beneficial.

The former rRNA homology group III organisms, represented by Comamonas acidovorans and C. testosteroni, have ATCases with molecular masses of approximately 500 kDa which are similar in size to the ATCase of B. pickettii (Table 36). These ATCases do not dissociate and are likely to remain members of class A.

During polyacrylamide gel electrophoresis and protein purification, the ATCase from C. testosteroni was shown to be extremely heat sensitive.

The ATCases from both species of Comamonas exhibited Michaelis-Menten saturation kinetics when one varied either aspartate or carbamoylphosphate. They had similar $K_m$ values for aspartate and carbamoylphosphate.

The nucleotide effector response to ATP or UTP showed 100 per cent inhibition for both Comamonas species at low or high levels of aspartate or carbamoylphosphate. At high
levels of aspartate or carbamoylphosphate, the ATCase of *C. acidovorans* was inhibited by CTP (Table 37 and 38).

*Brevunidimonas diminuta*, a former rRNA homology group IV organism, contained an ATCase similar in size to the class A 480 kDa ATCases and it did not dissociate into subunits (Table 36). The enzyme from *B. diminuta* exhibited Michaelis-Menten saturation kinetics when either aspartate or carbamoylphosphate concentration was varied.

The *B. diminuta* enzyme behaved like the class A ATCases except for its nucleotide effector response. There was no regulation by ATP, CTP, or UTP at low aspartate concentration. At high aspartate concentrations, there was no regulation by ATP or CTP but UTP gave 54% inhibition (Table 37). At low carbamoylphosphate levels there was 100 per cent inhibition by ATP, CTP, or UTP, but at high levels of carbamoylphosphate, there was no regulation by ATP or CTP, and 38 per cent inhibition by UTP (Table 38).

*Pseudomonas indigofera*, has never been assigned to a rRNA homology group and contains an ATCase with an Mr of approximately 400 kDa (Table 36). The *P. indigofera* enzyme produced a sigmoidal saturation curve when the aspartate concentration was varied. A $K_M^{[ASP]}$ value of 9.0 mM was observed. This was the lowest affinity for aspartate seen for any enzyme in this study. A Michaelis-Menten saturation kinetic curve was observed when the carbamoylphosphate was varied with a $K_M^{[CP]}$ value of 0.7 mM (Table 36). The results
obtained from this study, suggest that *P. indigofera* does not belong in the genus *Pseudomonas* as its ATCase does not fit the profile of a class A ATCase. The ATCase from this organism more closely resembles the class B ATCases even though the enzyme has a larger Mr of 400 kDa.

For the sake of analysis, I have chosen to discuss the ATCases of *Shewanella putrefaciens* and *Stenotrophomonas maltophilia* together as they have similar ATCases with molecular masses of 100 kDa. Both ATCase from these organism exhibited Michaelis-Menten saturation kinetics when the concentration of aspartate or the concentration of carbamoyl-phosphate was varied.

These results suggest that these enzymes belong to the class C ATCases. Shepherdson and co-workers have placed the *Stenotrophomonas maltophilia* enzyme in the class C based solely on molecular mass (63). However, the class C ATCases have no regulation by nucleotide effectors, whereas both 100 kDa enzymes in this study exhibited inhibition by nucleotide effectors (Tables 37 and 38).

A key question emerges regarding the source of the nucleotide binding sites for these two 100 kDa enzymes. The class C or class B trimers, which by definition have no nucleotide binding sites, could have acquired nucleotide binding sites. Or, this may represent the dissociation of a typical class A ATCase into active smaller subunits with the dissociated trimer fully active and having recruited the
capability to bind effectors perhaps as seen for the enzyme from wheat.

The overall results obtained from this study strongly support the idea that ATCase can be used as a taxonomic marker to aid in the realignment of the *Pseudomonas* genus. Table 40 lists the relationships of the genus to ATCase classification.
### TABLE 40. ATCase Classification as Compared to Genus Classification

<table>
<thead>
<tr>
<th>Previous Organism Name</th>
<th>Current Organism Name</th>
<th>rRNA Group</th>
<th>ATCase Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
<tr>
<td>Pseudomonas aureofaciens</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
<tr>
<td>Pseudomonas syringae</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
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<tr>
<td>Pseudomonas stutzeri</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
<tr>
<td>Pseudomonas mendocina</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
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<td>Same</td>
<td>I</td>
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<tr>
<td>pseudocaligenes</td>
<td>Same</td>
<td>I</td>
<td>Class A</td>
</tr>
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<td>Pseudomonas cepacia</td>
<td>Burkholderia cepacia</td>
<td>II</td>
<td>New ATCase Class Suggested</td>
</tr>
<tr>
<td>Pseudomonas pickettii</td>
<td>Burkholderia pickettii</td>
<td>II</td>
<td>Larger ATCase belonging to Class A</td>
</tr>
<tr>
<td>Pseudomonas acidovorans</td>
<td>Comamonas acidovorans</td>
<td>III</td>
<td>Larger ATCase belonging to Class A</td>
</tr>
<tr>
<td>Pseudomonas testosteroni</td>
<td>Comamonas testosteroni</td>
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<td>Larger ATCase belonging to Class A</td>
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<tr>
<td>Pseudomonas diminuta</td>
<td>Brevundimonas diminuta</td>
<td>IV</td>
<td>Class B</td>
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<td>Same</td>
<td>None</td>
<td>Larger ATCase belonging to Class B</td>
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<tr>
<td>Pseudomonas putrefaciens</td>
<td>Shewanella putrefaciens</td>
<td>None</td>
<td>Class C</td>
</tr>
<tr>
<td>Pseudomonas maltophilia</td>
<td>Stenotrophomonas maltophilia</td>
<td>V</td>
<td>Class C</td>
</tr>
</tbody>
</table>
REFERENCES


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