

PURIFICATION OF ASPARTATE TRANSCARBAMOYLASE FROM
MORAXELLA (BRANHAMELLA) CATARRHALIS

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The enzyme, aspartate transcarbamoylase (ATCase) from *Moraxella (Branhamella) catarrhalis*, has been purified. The holoenzyme has a molecular mass of approximately 510kDa, harbors predominantly positive charges and is hydrophobic in nature. The holoenzyme possesses two subunits, a smaller one of 40 kDa and a larger one of 45 kDa. A third polypeptide has been found to contribute to the overall enzymatic activity, having an approximate mass of 55 kDa. The ATCase purification included the generation of cell-free extract, streptomycin sulfate cut, 60 °C heat step, ammonium sulfate cut, dialysis and ion, gel-filtration and hydrophobic interaction chromatography. The enzyme's performance throughout purification steps was analyzed on activity and SDS-PAGE gradient gels. Its enzymatic, specific activities, yield and fold purification, were also determined.

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CHAPTER 1

THE ORGANISM UNDER STUDY

Microbiological characteristics of *Moraxella(Branhamella)catarrhalis*

Microbiological characteristics of *Moraxella (Branhamella) catarrhalis* reveal the organism as being a Gram negative bacterium frequently described as a very short and plump rod (1.0-1.5 μm wide by 1.5-2.5 μm in length) and occurring in pairs and short chains. The coccoid forms are usually smaller (0.6-1.0 μm in diameter) and occur as single cells or in pairs with the adjacent sides flattened, often called “coffee-bean” in shape (diplococci). The organism may be encapsulated, with no flagella present although surface-bound “twitching motility” has been observed in some rod-shaped species due to fimbriation. It is aerobic, requiring oxygen for growth, but some strains may grow weakly under anaerobic or anoxygenic conditions. Most species are nutritionally fastidious requiring enriched media for growth such as blood and chocolate agars, at an optimal temperature range of 33-35°C (Krieg, 1984). Colonies are not pigmented, are circular and opaque, non-endospore formers, are oxidase -positive and usually catalase positive. The organism produces no acid from carbohydrate metabolism and is nonhemolytic on blood agar. Most strains of *M.(B.) catarrhalis* produce DNase and reduce nitrate and nitrite (Koneman et al., 1994). The mol % G+C of the DNA ranges from 40.0-47.5 in

each of the two subgenera. The organism is usually highly sensitive to the antibiotic penicillin, although it has acquired resistance to such antibiotics and their relatives, e.g. cephalosporins, by producing beta-lactamases, enzymes that cleave the beta-lactam ring in these antibiotics, thus inactivating their antimicrobial action.

M.(B.) catarrhalis can be differentiated from other oxidase-positive, gram-negative cocci such as *Neisseria* spp., with tributyrine, 4-methylumbelliferyl butyrate (MUB) or indoxyl acetate as well as superoxol tests, giving positive reactions to all of them, while the *Neisseriaceae* are negative (Riley, 1987; Speeleveld et al., 1994). A multiplex PCR method for the detection of *Alloiococcus otitidis*, *Haemophilus influenzae*, *M. (B.) catarrhalis* and *Streptococcus pneumoniae* in middle ear effusions (MEEs) has been developed (Hendolin et al., 1997) and now modified to be better suited for clinical use (Hendolin et al., 2000).

Clinical significance

In clinical significance, *M.(B.) catarrhalis* is characterized in Bergey's manual as not being of high pathogenicity to man, and usually considered to be a harmless parasite of the mucous membranes of human beings and /or other animals, although most species may be opportunistic pathogens. Clinical reports have shown that the usual habitat of *M. (B.) catarrhalis* is the upper respiratory tract of healthy children (50.8%) and elderly adults (26.5%), whereas it is less common in healthy adults (1.5% to 5.4%) (Koneman et al., 1994). The coccus is most frequently isolated from the nasal cavity of man, its main natural habitat, the organism also inhabits the pharynx. Currently, *M.(B.) catarrhalis* is considered as the third most common pathogen of the respiratory tract after *Streptococcus*

pneumoniae and *Haemophilus influenzae*. It has been isolated from the inflammatory secretions of the middle ear (otitis media), maxillary sinus (sinusitis) in pediatric patients, and from bronchial aspirates in cases of bronchitis and pneumonia in adults having an underlying chest disease (Enright et al., 1997). Its main pathogenicity is reflected in low-grade otitis media of infants and respiratory complications in the immunocompromised host. In rare cases, it has been implicated in invasive infections such as meningitis, endocarditis, bacteremia, septic arthritis, osteomyelitis, epiglottitis, cellulitis, shunt-associated ventriculitis, peritonitis, pericarditis, wound infections and serious lower respiratory tract infections in neonates and infants. It is also an unusual cause of acute urethritis and conjunctivitis (Koneman et al., 1994). A study has been conducted of pathogens causing acute lower respiratory tract infection (LRTI) in hospitalized children (age less than 12 years) in Singapore, as a comparison of incidence between 1988 and 1995. Within the seven-year span the incidence of occurrence of bacterial causes of the illness was 36% in 1995 vs. 15% in 1988, and the most common bacteria found were *M.(B.) catarrhalis* (34.7%), non-type B *Haemophilus influenzae* (33%). The higher incidence of *M.(B.) catarrhalis* was thought to be attributed to antibiotic selection. Since in 1995, the percentage of penicillin resistance was found to be 83% in *M.(B.) catarrhalis* as compared to none in 1988 (Chong et al., 1997). Another study of patients between the ages of 21 to 73 years treated for chronic sinusitis, has revealed that 53% of the patients were infected with aerobic organisms including strains of *M.(B.) catarrhalis* (Radosz-Komoniewska et al., 1997). The most common cause of morbidity due to this organism is exacerbation of chronic bronchitis in adults with preexisting pulmonary

disease (Sarubbi et al., 1990). Acute otitis media (AOM) in children has been commonly attributed to such pathogens as beta-lactamase positive *Haemophilus influenzae* and *M. (B.) catarrhalis*, penicillin-resistant *Streptococcus pneumoniae* and penicillin-susceptible *S. pneumoniae*. Another pathogen, usually associated with pediatric pneumonia, called *Chlamydia pneumoniae* has been for the first time recovered from middle ear fluid of children with AOM with a frequency of 8% (Block et al., 1997). Incidentally, another species from the genus *Moraxella* has been isolated in large numbers from an ulcerated supraclavicular lymph node of a terminal patient, *Moraxella canis*. This organism is an upper-airway commensal from dogs and cats and is considered nonpathogenic in humans, but its third incidence in humans changes this notion (Vanechouette et al., 2000). Therapeutic agents have been applied in treatments of infections caused by *M.(B.) catarrhalis* and other upper respiratory pathogens with mixed success as the following examples reveal. The frequency of beta-lactamase production was analyzed in 1452 strains of *M.(B.) catarrhalis* and 2738 strains of *H. influenzae* isolated from middle ear fluid of Finnish children between the ages of 1 to 6 years between 1978 and 1993 as well as 1988 to 1990. The findings revealed a marked increase of *M.(B.) catarrhalis* strains producing beta-lactamase, from 0-60% in 1978-1983 and from 60-80% in 1988-1990, that correlated with increased consumption of beta-lactam antimicrobials, such as cephalosporin, within communities (Nissinen et al., 1995). The antibacterial activity of RU 64004, a new ketolide, was evaluated against more than 600 bacterial strains and was found to inhibit 90% of pneumococci resistant to erythromycin A and penicillin G, as well as other bacteria involved in respiratory infections including

H. influenzae, *M.(B.) catarrhalis* and *Streptococcus pyogenes* (Agouridas et al., 1997). In view of the rising resistance to the penicillin family of antibiotics among many respiratory pathogens producing beta-lactamases, a new expanded spectrum oral cephalosporin, ceftibuten, has been synthesised and found to be effective against many gram negative organisms, such as *M.(B.) catarrhalis*, as well as selected gram-positive organisms (Guay, 1997). A study of patients with respiratory tract infections revealed that resistance to ampicillin was found in 66.7% of strains of *M.(B.) catarrhalis*, of which over 70% of them were producers of beta-lactamase. All strains were sensitive to ofloxacin and amoxicillin/clavulanic acid (Jakubicz et al., 1997). A new 8-methoxy fluoroquinolone antimicrobial, gatifloxacin, was tested against 1400 strains of *H. influenzae* and 600 strains of *M.(B.)catarrhalis* and was found to be most active against *H.influenzae* and was similar in action to four comparison fluoroquinolones against *M.(B.) catarrhalis* (Jones et al., 1999).

Virulence factors

As an important respiratory pathogen, *M.(B.) catarrhalis* is armed with numerous virulence factors to allow its invasion and colonization of human respiratory tract. Several of these factors have been identified and characterized in the last decade including outer membrane proteins (OMPs), lipooligosaccharides (LOS), beta-lactamase, iron-proteins, hemagglutinins and hemolysins, pilin and tetrameric repeat units. The presence of a capsule is possible and may prove to be another virulence factor in *M.(B.) catarrhalis* (Ahmed et al., 1991). Additionally, the bacterium produces transferrin- and lactoferrin-binding proteins enabling it to acquire iron from its environment, and some strains have

been found to be serum resistant. All these factors taken into consideration, together with the existence of three serotypes of lipopolysaccharide and a rising trend in nosocomial spread of the organism in hospital respiratory ward, places *M.(B). catharrhalis* within the category of emerging respiratory pathogens (Enright et al, 1997). The means of helping to invent more efficient methods of treatment, prevention and control of this organism are markedly diminished without further study that would lead to the understanding of its pathogenic properties, its relationship with other respiratory pathogens, both on the level of virulence as well as phylogenetic relatedness, and finally, its genetic and molecular makeup.

OUTER MEMBRANE PROTEINS

There are several outer membrane proteins (OMPs) identified so far in *M.(B). catharrhalis* including UspA1 and A2, OMP B1, OMP B2(CopB), OMP CD and OMP E.

The UspA protein is present on the surface of all *M.(B). catharrhalis* disease isolates and therefore constitutes a target for monoclonal antibodies (Aebi et al., 1994 and 1997). The corresponding gene, *uspA1*, encodes a protein of approximate molecular mass of 88, 271 Da while a second gene, *uspA2* encodes a protein of smaller dimensions, 62,483 Da. However, these proteins share a 140-amino acid region with 98% identity. Both proteins act as antigens binding specific monoclonal antibodies and most closely resembled adhesins expressed by other bacterial pathogens (Aebi et al., 1997). Study using mutants of a *M.(B). catharrhalis* strain indicated that UspA1 was involved in adherence to

Chang conjunctival cells in vitro, while the expression of UspA2 on cellular surface conferred to *M.(B). catarrhalis* resistance to killing by normal human serum (Aebi et al., 1998). Further study has determined the N-terminal amino acid sequence of the mature forms of the UspA1 and UspA2 proteins as well as the nucleotide sequence of the corresponding genes. The nucleotide sequence revealed that the two protein products of the *uspA1* and *uspA2* genes showed very close similarity between other *M.(B). catarrhalis* strains as compared to the one used in this study, as well as exhibited several different and repetitive amino acid motifs, including some coiled coil forms (Cope et al., 1999). In another study, the protein encoded by *uspA2* gene from three *M.(B). catarrhalis* strains, showed that in two strains at least, the N-terminal half of the protein UspA2 resembled the N-terminal half of UspA1 protein. The C-terminal half of this protein was found to be nearly identical to previously characterized UspA2 proteins. The researchers in this study designated the “hybrid” protein as UspA2H, and found that about 20% of *M.(B). catarrhalis* strains apparently possess a *uspA2H* gene instead of a *uspA2* gene. The *M.(B). catarrhalis uspA1, uspA2 and uspA2H* genes have been cloned (Lafontaine et al., 2000).

The OMP B1 has been discovered in patients with bronchiectasis (chronic dilation of a bronchus or bronchi, with a secondary infection that usually involves the lower portion of the lung) who were persistently colonized with *M.(B). catarrhalis*. The predominant antibody response was to a 84,000 Da outer membrane protein, OMP B1, on the surface of *M.(B). catarrhalis* and it was heterogenous among strains of this organism, designating it as an important antigenic candidate (Sethi et al., 1995). Further study has

demonstrated that OMP B1 binds human transferrin, and is conserved in the outer membrane of all iron-stressed clinical isolates of *M.(B). catarrhalis* and therefore this protein might be involved in iron-uptake mechanism utilized by this bacterium (Campagnari et al., 1996). Another study has successfully cloned *ompB1* gene, and sequence analysis has suggested that OMP B1 in *M.(B). catarrhalis* is a homologue transferrin-binding protein B in pathogenic *Neisseria* spp., *H. influenzae*, *Actinobacillus pleuropneumoniae* and other *M.(B). catarrhalis* strains, further corroborating previous study of the iron-sequestering role of this protein and its conservation among bacterial pathogens of the respiratory tract. The study also showed that 31% of clinical isolates tested expressed the OMP B1 epitope, suggesting its use as a potential vaccine antigen against *M.(B). catarrhalis* (Luke et al., 1999).

The OMP B2 or Cop B outer membrane protein in *M.(B). catarrhalis* has been found to be of approximate molecular mass of 80,000 Da, and the antibody against it bound to 70% of *M.(B). catarrhalis* strains tested. The gene encoding Cop B has been cloned in *Escherichia coli*, and its nucleotide sequence revealed that the protein was synthesised with a leader peptide, further confirmed by N-terminal amino acid sequence analysis of the mature Cop B protein. The extent of conservation of this protein among *M.(B). catarrhalis* strains has been established by showing that a probe comprising the majority of *copB* structural gene from a strain used in the study, hybridized with seven different *M.(B). catarrhalis* strains, and conversely a polyclonal antisera raised against Cop B proteins from different *M.(B). catarrhalis* strains used to probe the recombinant

form of the Cop B protein from the strain used in the study (Helminen et al., 1993). Further study has revealed that Cop B might be involved in the iron-uptake mechanism (just as is OMP B1). Wild-type strains of *M.(B). catarrhalis* were found to be able to utilize ferric citrate, transferrin, lactoferrin and heme as sources of iron for growth *in vitro*, while an isogenic mutant of CopB was severely impaired in its ability to utilize transferrin and lactoferrin as sole sources of iron, although it could utilize ferric citrate for growth. These studies indicate that without Cop B protein being expressed, the organism has difficulty to utilize iron bound to certain carrier proteins, such as transferrin (Aebi et al., 1996). Another study has proven that *copB* gene is largely conserved among strains of *M.(B). catarrhalis*, by showing 92-95% homology between sequences at the nucleotide level and 90-95% homology at the amino acid level for two different strains of *M.(B). catarrhalis*. Restriction Fragment Length Polymorphism (RFLP) analysis of *copB* genes from 20 diverse *M.(B). catarrhalis* strains, revealed 90% of potential restriction sites in the constant and 47% of potential restriction sites in variable regions, respectively, classifying it as a general pattern among strains of *M.(B). catarrhalis* (Sethi et al., 1997).

The OMP CD of *M.(B). catarrhalis* has been found to be a major outer membrane protein and its gene DNA sequence determined. Sequence analysis has revealed a high degree of homogeneity in the *ompCD* gene between strains of *M.(B). catarrhalis*, with minor sequence heterogeneity in three regions of the gene, again proving the conservation of the gene among clinical isolates of this pathogen (Hsiao et al., 1995).

Further study has elucidated the molecular mass of the OMP CD protein to be 45-kDa, whose antigenic structure revealed two-surface exposed epitopes. Sera from adult patients

suffering from chronic obstructive pulmonary disease (COPD) contained antibodies which recognized excessively the peptide corresponding to the central region of the OMP CD molecule, thus making it a likely target for a vaccine against *M.(B). catarrhalis* (Murphy et al., 1999).

The OMP E of *M.(B). catarrhalis* has been found to be a 50-kDa and a potential vaccine candidate. The OMP E gene has been cloned and shown to have an open reading frame (ORF) of 1377 bp encoding a protein of 460 amino acids, with an estimated molecular mass of 47,030 Da. The protein exhibits borderline homology to the Fad L protein of *Escherichia coli* (49.1%) which is involved in the binding and transport of fatty acids, as well as being related to porins of the OMP F family. RFLP analysis proved high conservation of the OMP E gene among 19 different strains of *M.(B). catarrhalis* (Bhushan et al., 1994). Further studies have elucidated the antigenic structure of OMP E, with the identification of three surface –exposed epitopes recognized by specific monoclonal antibodies on the surface of *M.(B). catarrhalis*. One particular epitope has been identified to comprise the 80-180 lactamases that have been found in in *M.(B). catarrhalis*, named BRO-1 and BRO-2 (Sanders & Sanders, 1992). Extrapolating on that finding, one beta-lactamase is a stronger acting BRO-1 enzyme and the other weaker-acting BRO-2 enzyme. Seventy strains of *M.(B). catarrhalis* have been used in the study, among which 59 strains showed beta-lactamase production, specifically BRO-1 enzyme activity was identified in 55 strains, BRO-2 enzyme activity in 3 strains and none in one strain. However, all strains showed susceptibility to amoxicillin/clavulanic acid combination despite the presence of these enzymes, while none of the beta-lactamase

producing strains were susceptible to penicillin and ampicillin (Ejlertsen et al., 1996).

Further studies have shown that BRO is expressed as a 33-kDa lipoprotein associated with the inner leaflet of the bacterium's outer membrane (Bootsma et al., 1999).

IRON-PROTEINS

It has been shown that *M.(B). catarrhalis* can obtain iron from human transferrin and lactoferrin iron-carrier proteins, for growth requirements. When it is stressed for iron availability, the organism expresses new outer membrane proteins that are not detected in growth conditions enriched with iron. These proteins have been characterised as being iron-repressible (Campagnari et al., 1994). A more detailed recent study has identified these proteins as transferrin-binding receptor protein (Tbp B or OMP 1) and lactoferrin-binding protein B (Lbp B), to which antisera from convalescent and acute patients with pulmonary infections, reacted most strongly. These particular proteins can thus serve as potential vaccine candidates against *M.(B). catarrhalis* (Rong-hua et al., 1999).

HEMAGGLUTININS/HEMOLYSINS

A study has shown that strains of *M.(B). catarrhalis* which were associated with respiratory tract infections in the elderly, were found to be agglutinating human red blood cells, as well as having shown resistance to complement killing in normal human serum (NHS), as compared to strains from healthy elderly patients (Murphy et al., 1997; Fitzgerald, 1997). *Moraxella bovis*, which is the causative agent of bovine keratoconjunctivitis, was shown to have hemolytic activities associated with production of a 94-kDa protein, with endotoxin properties (the hemolytic action requires a binding step

and Ca^{2+} ions similar to RTX activity of bacterial endotoxins), therefore suggesting that these two activities are either related or have common epitopes in this organism (Billson et al., 2000).

PILI

Bacteria that colonise mucosal surfaces express pili on their surfaces for attachment, protein filaments that are made of pilin protein (Marrs et al., 1990). Pili have not been linked conclusively with virulence in *M.(B). catarrhalis* but they have been in *M. lacunata* and *M. bovis* (Atwell et al., 1994; Heinrich et al., 1997).

To conclude on the array of *M.(B). catarrhalis* virulence factors, it has been found that tetrameric repeat units such as 5'-CAAT-3' are linked with phase variable expression of lipopolysaccharide biosynthetic genes in such respiratory pathogens as *H. influenzae*, *Neisseria* spp. and *M.(B). catarrhalis*. In *M.(B). catarrhalis*, a repeated motif 5'-CAAC-3' is probably associated with the same genes as in *H. influenzae*, which have been suggested as being putative virulence factors (Peak et al., 1996).

Nomenclature

Since its discovery at the end of the nineteenth century, the taxonomic relationship of *M.(B). catarrhalis* and other related genera remains controversial, resulting in frequent changes in nomenclature of this bacterium's name as well as its standing as either a commensal or a pathogen. Ghon and Pfeiffer (Koneman, 1994) first described the organism and called it *Micrococcus catarrhalis*. The genus name was then changed to *Neisseria catarrhalis* because of the organism's phenotypic and ecological similarities to *Neisseria* spp. In 1970 *Moraxella catarrhalis* was transferred from genus *Neisseria* to

Branhamella on the basis of differences in fatty acid content and DNA hybridization studies compared with other members of the *Neisseria* spp. (Catlin, 1990). The latest proposed scheme places *Branhamella* as a subgenus of *Moraxella*, such that: rod-shaped organisms or *Moraxella* in one species and cocci or *Branhamella* in a separate species, and based on a distinction that *Branhamella catarrhalis* is a common human respiratory pathogen, whereas *Moraxella* spp. constitutes unusual human pathogen (Catlin, 1970; Bovre, 1979).

According to the 1984 Bergey's Manual of Systematic Bacteriology (Krieg, 1984) classification scheme, the *Neisseria*, *Kingella*, *Acinetobacter* and *Moraxella* species are placed within the *Neisseriaceae* family. The 1989 version proposed a scheme of *Naiseriaceae*, where the species of *Acinetobacter* and *Moraxella* are not included, because in both cases family affiliation is uncertain. Separate families are proposed instead, those of *Moraxellaceae* (Rossan et al. 1991); based on DNA-ribosomal RNA hybridization studies, and *Branhamellaceae* (Catlin, 1991). More recent studies based on 16S ribosomal RNA sequence data, have further confirmed the earlier proposed scheme that the genera *Acinetobacter* and *Moraxella* and [*Neisseria*] *ovis* should be excluded from the family *Neisseriaceae*. These excluded taxa, consistent with hybridization data, should be part of a new family, the *Moraxellaceae* (Enright et al, 1994). The family of *Moraxellaceae* is further subdivided into four phylogenetic groups, where *Moraxella* (*Branhamella*) *catarrhalis* is placed into Group I, the classical *Moraxellae* of the coccal morphology, all based on 16S RNA sequence similarities. Group II contained *M. atlantae* and *M. osloensis*, Group III contained strains of the genus *Psychrobacter* and strain 752/52 of [*Moraxella*]

phenylpyruvica, while Group IV contained strains of the genus *Acinetobacter* (Pettersson et al., 1998). Using sequence analysis of PCR-amplified bacterial small subunit 16S RNA genes, strains from deep seawater formed a new separation within the *Moraxella*-*Psychrobacter* branch of the *Moraxellaceae* family, revealing a disparity between terrestrial and Antarctic relatives (Maruyama et al., 2000). Bergey's manual places *Moraxella catarrhalis* within the subgenus *Branhamella* (Catlin, 1970; Bovre, 1979) such that the organism's current genus name is *Moraxella (Branhamella) catarrhalis* (Koneman, 1994).

CHAPTER 2

THE PROTEIN UNDER STUDY

Introduction

Concern about disputed nomenclature and thus proper phylogenetic placement, has stimulated researchers to employ tools and methodologies, such as biochemical properties, ribosomal RNA, phenotypic characteristics, numerical taxonomy and metabolic pathways to name a few, in order to show relatedness among bacteria (Stanier, et al., 1966). When an essential enzyme is found among all microorganisms it can be safely presumed that it has originated from a common ancestor, and that it has retained its basic physiological characteristics (although structural characteristics have changed) and regulatory properties across evolutionary time. Such an enzyme can thus serve as a taxonomic tool (Canovas et al., 1967; London, 1977). Aspartate transcarbamoylase (ATCase) has been shown to be useful in detecting relatedness among members of such families as the *Enterobacteriaceae*, *Yersinia enterocolitica* and *Y. enterocolitica*-like organisms (Folterman, 1978). The ATCase from *M.(B). catarrhalis* is an example of such a conserved protein.

The long term goal of this study is to use ATCase as a taxonomic tool in order to help resolve the controversy of this microorganism's correct placement within the bacterial phylogenetic tree. The immediate goal of this project was to purify and derive an amino acid sequence of the N-terminus of this enzyme, with a further plan to map the

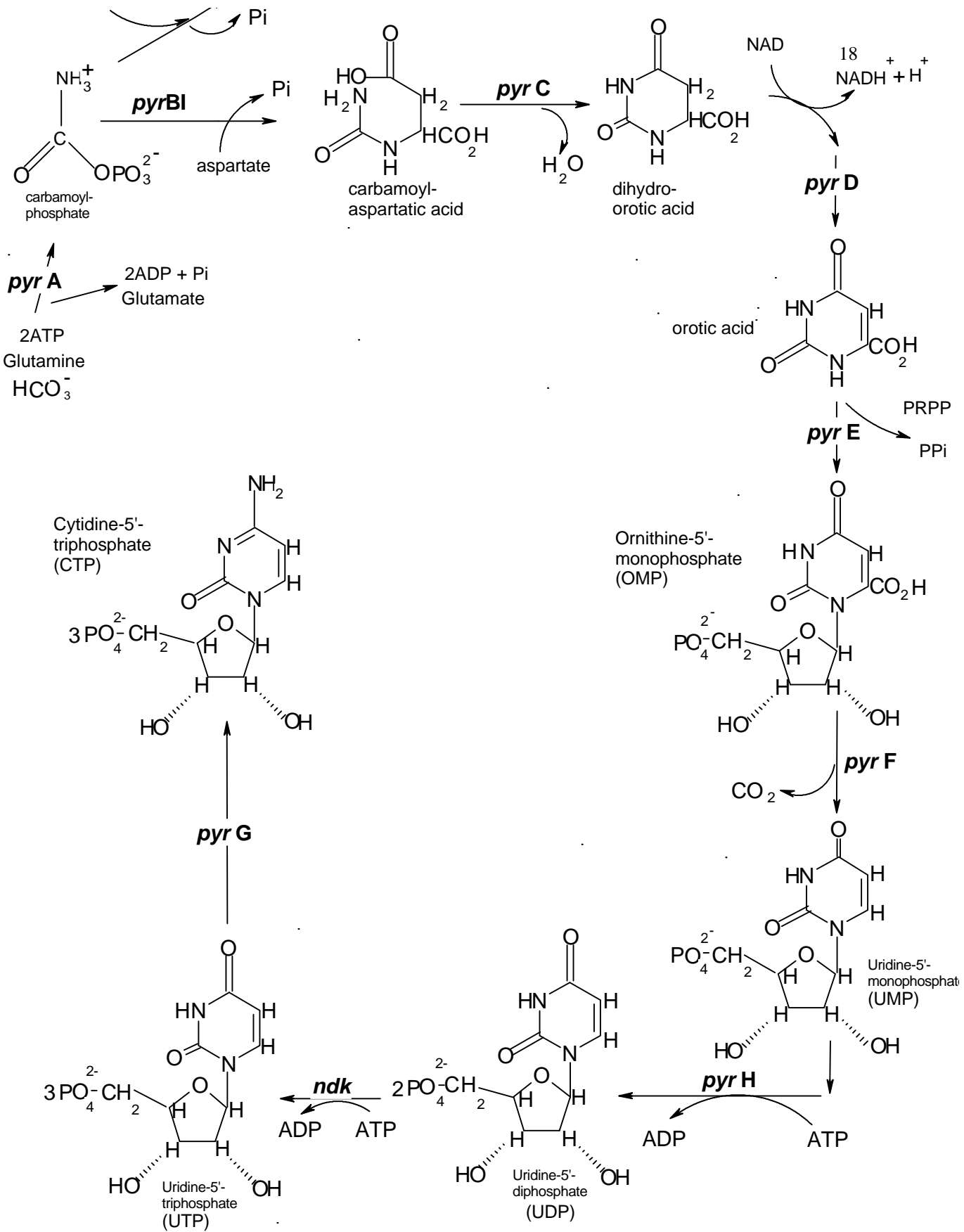
corresponding *pyrB* gene onto the *M. (B). catarrhalis* genome. Previously, the research objective was to clone and derive a nucleotide sequence of the *pyrB* gene in this microorganism with an attempt to perform evolutionary analysis and comparison with previously characterized ATCases from other organisms.

Pyrimidine Biosynthesis- Role of ATCase in Living Cells

The *de novo* biosynthesis is an important biochemical pathway in living organisms, whence it leads to the production of pyrimidine nitrogenous bases including uracil (U), thymine (T) and cytosine (C). Pyrimidines are found in all organisms, and structurally are six-membered aromatic heterocyclic ring compounds. They constitute components of pyrimidine nucleosides, each type consisting of one of the nitrogenous bases (either U, T or C) linked covalently to a pentose sugar. With an additional phosphate group attached to the previous nucleoside, a nucleotide is created. Pyrimidine nucleotides, together with purine nucleotides (that include nitrogenous bases adenine (A) and guanine (G)), comprise the backbone of nucleic acids in all living cells (deoxyribonucleic acid or DNA, and ribonucleic acid or RNA). The biosynthesis of uridine-5'-mophosphate (UMP) serves as a precursor for all pyrimidine nucleotides, UTP and CTP, and is accomplished via the pyrimidine biosynthetic pathway. This pathway appears to be present in all organisms including bacteria, fungi, plants and animals. It has been most extensively studied in *Escherichia coli*, *Salmonella typhimurium* and other enteric bacteria (O'Donovan & Neuhard, 1970; Grogan & Gunsalus, 1993), while the non-enteric bacteria include members of the genus *Bacillus* spp. and *Pseudomonas* spp.

FIGURE 2.1 THE *DE NOVO* PYRIMIDINE BIOSYNTHETIC PATHWAY IN BACTERIA, IN *Escherichia coli*.

The names for the abbreviations marked in bold are as follows: ***pyrB***, aspartate transcarbamoylase (ATCase, EC2.1.3.2); ***pyrC***, dihydroorotase (DHOase, EC3.5.2.3); ***pyrD***, dihydroorotate dehydrogenase (DHODEHase, EC1.3.3.1); ***pyrE***, OMP phosphoribosyltransferase (OPRTase, EC2.4.2.10); ***pyrF***, OMP decarboxylase (OMPdecase, EC4.1.1.23); ***pyrH***, UMP kinase (EC2.7.4.4); ***ndk***, nucleoside kinase (EC2.7.4.6); and ***pyrG***, CTP synthase (EC6.3.4.2).



The arginine pathway in *E. coli* (Figure 2.1) competes with the pyrimidine pathway for the substrate carbamoylphosphate (Pierard et al., 1965; Makoff & Radford, 1978; Stalon et al., 1987; Wong & Abdelal, 1990). The pyrimidine biosynthetic pathway is controlled by an array of enzymes that are encoded by their structural genes. The first enzyme in the *de novo* pyrimidine biosynthesis and arginine biosynthesis is carbamoylphosphate synthetase ; (EC 6.35.5; CPSase; encoded by genes *carAB* or *pyrA*). This enzyme catalyses the formation of carbamoyl phosphate from the amine group of amino acid glutamine, bicarbonate and ATP. The following step is unique to pyrimidine synthesis because it is the first committed step leading to the outcomes of this pathway, the pyrimidine nucleotides. It is catalysed by aspartate transcarbamoylase (EC2.1.3.2; ATCase; L-aspartate carbamoyl transferase; encoded by the *pyrBI* gene). ATCase condenses carbamoylphosphate with the amino group of aspartate and forms N-carbamoyl-L-aspartate and inorganic phosphate (Pi). In the third step, dihydroorotate is formed as a result of cyclizing of carbamoyl-L-aspartate with a loss of one water molecule. This reaction is catalysed by the enzyme dihydroorotase (EC 3.5.2.3; DHOase; encoded by the *pyrC* gene) (Baeckstrom et al., 1986). In the fourth step of the pathway, dihydroorotate is oxidised by dihydroorotate dehydrogenase (EC 1.3.3.1; DHOdase; encoded the *pyrD* gene) (Larsen & Jensen, 1985) to form orotate (first pyrimidine). In the next reaction, orotate phosphoribosyltransferase (EC 2.4.2.10; OPRTase; encoded by *pyrE* gene), catalyses the condensation of orotate with 5-phosphoribosyl-1-pyrophosphate (PRPP) to yield pyrophosphate and orotidine-5'-monophosphate (OMP), the first pyrimidine nucleotide (Poulsen et al., 1983). The following reaction, catalysed by OMP

decarboxylase (EC 4.1.1.23; OMPdecase; encoded by the *pyrF* gene), uridine-5'-monophosphate (UMP) is produced by decarboxylation of OMP.

Next, UMP kinase (EC 2.7.4.4; encoded by *pyrH* gene), catalyses the phosphorylation of uridine-5'-diphosphate (UDP) by UMP with the use of ATP as energy source.

Subsequently, uridine-5'-triphosphate (UTP) is synthesized from the phosphorylation of UDP by the enzyme nucleoside diphosphate kinase (EC 2.7.4.6; encoded by the *ndk* gene), with ATP as energy source (Ginther & Ingraham, 1974). Finally, the enzyme cytidine-5'-triphosphate (CTP) synthetase (EC 6.3.4.2; encoded by *pyrG*) catalyses the amination of UTP to CTP.

Classification of ATCase Enzymes

As mentioned previously, the ATCase enzyme can be found in all organisms, both eucaryotic and procaryotic. In the procaryotes, ATCases are separated into three classes according to their molecular sizes and regulatory properties (Bethell & Jones, 1969). The three known classes of bacterial ATCases, are designated as class A, B and C, respectively (Figure 2.2):

Class A ATCases are found in *Pseudomonas*. The holoenzyme has a molecular mass of 470-500 kDa. Structurally it exists as a dodecamer, composed of six *pyrB*-encoded 34-kDa polypeptides associated with six *pyrC*'- encoded 45-kDa polypeptides. No *pyrI*-encoded regulatory polypeptide has been found in this class of ATCases. The active site of the enzyme and the binding site for nucleotide effectors was found to be located on the 34-kDa polypeptide (Bergh and Evans, 1993). In *Pseudomonas putida* for example, the

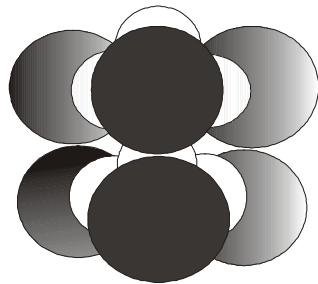
regulatory nucleotide binding site is found within the N-terminal extension of the 34-kDa polypeptide (Bergh & Evans, 1993). A second gene in the *P. putida* ATCase holoenzyme complex, *pyrC'* which encodes the 45-kDa polypeptide, has significant homology to dihydroorotase (DHOase) from other organisms, but has no DHOase activity. This inactive *pyrC'* (which in other bacteria encodes DHOase) has been designated *pyrC'* with a proposed function of assuring ATCase activity, by conserving the dodecameric assembly of the native enzyme (Schurr et al., 1995). Saturation curves for carbamoylphosphate and aspartate substrates, are hyperbolic, becoming sigmoidal when UTP, is used. ATP also strongly inhibited the enzyme (Neumann & Jones, 1964).

Class B ATCases are found in *Escherichia coli*. The holoenzyme has a total mass of 310 kDa. It is a dodecamer, $2B_3:3I_2$, composed of two catalytic trimers (B_3) made of six *pyrB*-encoded 33-kDa identical polypeptides, associated with three regulatory dimers (I_3) made up of six *pyrI*-encoded 17-kDa polypeptides. The *pyrB* gene encodes a 311 amino acid- long catalytic polypeptide. The *pyrI* gene encodes a 153 amino acid- long regulatory polypeptide (Kantrowitz & Lipscomb, 1988). The catalytic polypeptide has two domains, where the carbamoyl phosphate binding region is found in the amino terminus (polar) domain and the aspartate binding region is found in the carboxy terminus (equatorial) domain. The regulatory polypeptide also has two domains, the effector binding sites for ATP and CTP, which are located in the amino terminus, and a zinc binding site found at the carboxy terminus domain (Weber, 1968). Six zinc molecules stabilize the regulatory dimer and promote the association of the regulatory and catalytic subunits (Markby et al., 1991). This particular holoenzyme structure is conserved among

all enteric ATCases. *E.coli* ATCase is inhibited by CTP and is activated by ATP, with a sigmoidal dependence on substrate concentration at an optimum pH of 7.0.

Class C ATCases are found in *Bacillus subtilis*. The holoenzyme has a molecular mass of 100-kDa, is composed of three identical chains forming catalytically active trimers, each a 34-kDa polypeptide chain, encoded by *pyrB* gene. It displays hyperbolic kinetics for both substrates, aspartate and carbamoyl phosphate, and is not regulated by nucleotide effectors such as ATP,CTP or UTP (Brabson & Switzer, 1975; Maurizi & Switzer, 1978).

Figure 2.2. Classification of bacterial ATCases.

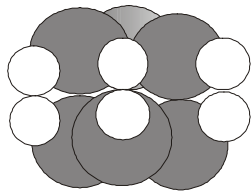


CLASS A ATCases Mr~480 kDa

Catalytic trimer of 34kDa
(One polypeptide, *pyrB*)



Polypeptide of 45kDa
(*pyr C'*)



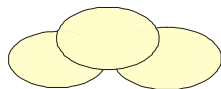
CLASS B ATCases

Mr~ 310kDa

Catalytic trimer
Of 34 kDa(*pyrB*)
(One polypeptide)



Regulatory dimer
Of 17 kDa (*pyrI*)



CLASS C ATCases

Mr~ 100 kDa

Catalytic trimers
Of 34kDa
(One polypeptide)
pyrB



It is important to note that the ATCase *pyrB* gene's nucleotide sequence, its protein amino acid makeup, the holoenzyme's structure and activity in various microorganisms has already been elucidated in *Escherichia coli*, *Pseudomonas putida* (Holloway et al., 1990; Schurr et al. 1992, 1995) and the eucaryotic CAD enzyme complex, such as in the fruit fly *Drosophila melanogaster* and yeast, *Saccharomyces cerevisiae* (Greyson et al., 1985). Other genes implicated in the initial steps of the pyrimidine pathway, such as *pyrI* (involved in regulatory functions of *pyrB*); *carAB* (encoding carbamoyl phosphate synthetases or CPSase) and *pyrC* (encoding dihydroorotase or DHOase) are studied in conjunction with ATCase *pyrB* because the genes that encode them together with the gene for ATCase, have been found either to manifest fused activities or overlap one another or affect the expression and assembly of their respective protein products (this is especially true of the *pyrI* and *pyrC* genes) (Schurr, 1992; Vickrey, 1993). Therefore, these organisms can serve as reference material for the study of ATCase in *M. (B). catarrhalis* in view of the fact that so far a little over 10 various gene sequences has been identified and cloned in this bacterium compared to the four thousand genes cloned for *Escherichia coli*, but their number is steadily increasing.

***Moraxella (Branhamella) catarrhalis* ATCase**

Research on the *de novo* pyrimidine biosynthetic pathway for *M.(B). catarrhalis* has been scanty, though it has been documented that the effector CTP is a strong inhibitor, while ATP or GTP have no stimulating effect on the pathway (Jyssum, 1992). A

more recent study on the ATCase enzymatic component of this pathway in *M.(B). catarrhalis*, has revealed that the ATCase in this bacterium has a pH optimum of 9.5 or higher. The enzyme is stable at 60°C for five minutes, is soluble at 25% ammonium sulfate saturation and precipitates at 60% saturation. It is bound to DEAE cellulose column at a pH of 8.5 in 50 mM salt buffer and elutes completely with a linear 50 to 500 mM salt gradient. The enzyme exhibited hyperbolic saturation with the substrate aspartate, while no allosteric response to increasing concentration of the substrate was detected. Its Michaelis-Menten kinetic response to substrate aspartate, has a K_m of 2 mM. The holoenzyme has an approximate molecular mass range of 480-520kDa, is arranged as a dodecamer composed of six 35-kDa subunits and six 45-kDa subunits (Fowler, 1998).

CHAPTER 3

MATERIALS AND METHODS

Experimental Outline

Preparation of cell-free protein extracts from bacterial cells; purification of the enzyme aspartate transcarbamoylase (ATCase). Purification steps include a streptomycin sulfate cut, a 60°C water bath, ammonium sulfate cut and dialysis. Column chromatography purification method has been shown to be best in the order as follows: ionic, gel filtration and hydrophobic interaction. Finally, electroblotting onto PVDF would be used to immobilize the protein for N-amino-terminus sequencing. The amino-acid sequence would be used for oligonucleotide probe generation in order to map the *pyrB* gene on the *M.(B.) catarrhalis* chromosome.

Chemicals and Media

N-carbamoyl-L-aspartate, L-aspartic acid potassium salt (Sigma®), acetic acid, acrylamide (BioRad®), agar granulated (Difco®), agarose (Fisher ®), ammonium sulfate, ammonium persulfate, bis-acrylamide (BioRad®), Blue Dextran 2000, Bovine Gamma Globulin STD(BioRad®), Brain Heart Infusion (Bacto®), bromophenol blue, carbamoylphosphate (Sigma®), Coomassie blue R-250, 1,5-dimethyl-2-phenyl-3-pyrazone (antipyrine)(Sigma®), betamercaptoethanol, 2,3-butanedione monoxime, diethanolamine, Ethylenediaminetetracetic acid (EDTA), FILDES Enrichment medium (Difco®), glacial

acetic acid, glycine, glycerol, N-ethylmorpholine, L-histidine, hydrochloric acid, lead nitrate, MES, methanol, phosphoric acid, sodium chloride, sodium dodecyl sulfate (SDS), sodium hydroxide; streptomycin sulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), Tris-base (hydroxymethylaminomethane), zinc sulfate; PVDF Protein Sequencing Membrane, 0.2 μ m pore size (BioRad). Distilled water was used in all experiments requiring it.

Materials and Equipment

Deep-freezer (Puffer-Hubbard™); Isotherm Incubator 30/37°C (Fisher-Scientific); Isothemp202 waterbath (Fisher Scientific); 96-well Microplates (Nunclon™); Bio-Rad DC Protein Assay Kit; Quantum Prep® Plasmid Mini and Maxiprep Kit (Bio-Rad); V-max Kinetic Microplate Reader (Molecular Devices); Soft-Max Software, Ver-3.25 by Dale; Vacuum pump (Precision Scientific); Microfiltration systems (MFS); Refrigerated centrifuge, EPPENDORF 5403; EPPENDORF microcentrifuge, model 5415C; Fisher Biotech Submarine Electrophoresis Systems, Minigels Horizontal Units, Fisher Scientific; GelDoc 1000-PC, BIORAD, Molecular Analysts Software/PC Gel documentation Software, Ver 1.4; Chromatography columns from Pharmacia Biotech included, Q Sepharose® Anion Exchange HP XK 16/20, size-exclusion Superdex 200 HL XK 26/60 and Sephacryl S-300 HR, hydrophobic interaction Phenyl Sepharose® 6Fast Flow (low sub); GradiFrac System with HiLoad Pump P-50 and UV-1 Monitor from Pharmacia Biotech; PerkinElmer Lambda 3A UV/V18 Spectrophotometer; Analytical balance (Sartorius); Top loading balance (OHAUS®); French press cell (SLM Aminco, Urbana,

ILL); Millipore UltraFree®-4 Centrifugal Filter Unit concentrators; Spectra/Por® Cellulose-Ester dialysis membrane, MWCO 15,000(Spectrum®); Lab Line Junior Orbit Shaker, Cat No.3520; Precision Glide®becton Dickinson & Co. 10mL, 5mL, 3ml and 1mL syringes; Pyrex® Glassware; Gel Doc 1000 Gel Photography (BioRad).

Bacterial Strains

Moraxella (Branhamella) catarrhalis American Type Culture Collection strain 25240 used in all protein experiments.

Methods

GROWTH OF CELLS, HARVESTING OF CELLS AND PREPARTION OF CRUDE PROTEIN EXTRACT

A fresh Brain-Heart Infusion (BHI) agar plate was streaked with the *Moraxella(Branhamella) catarrhalis* strain 25240 from the -80 °C stock. The plate was incubated in the 37 °C incubator for 12-18 hours. Few selected colonies were subcultured onto another BHI agar plate and streaked for individual colony isolation. The plate was incubated as above for 12-18 hours. A Gram stain was performed on one well isolated colony and observed under a microscope 1000 X power objective. It revealed scattered Gram negative diplococci, confirming that the organism is indeed *M.(B).catarrhalis*. A 25 mL of sterile BHI broth was inoculated with 1-2 well isolated colonies from the pure culture plate and incubated in a 250 mL screw-cap Enlenmeyer flask in a 37 °C incubator-shaker, at 250 rpm speed. After 12-18 hours, the flask was used as a starter culture to seed four individual 4 L culture flasks, each containing 2L of sterile BHI broth. Two mL

of the starter culture was added to each flask, constituting a 1/1000 dilution. The four flasks were incubated at 37 °C incubator-shaker, 18-24 hours at shaker speed of 250 rpm. The growing bacterial culture attained concentration of cells per mL approximating 1.602×10^9 cells/mL, deduced from an Optical Density or O.D. at 600 nm corresponding to 1.602 ($0.1 \text{ O.D} \cong 10^8$ cells/mL). The cells were pelleted by transferring the cell culture into four 500 mL capacity centrifuge bottles, balanced and centrifuged at 10,000 g for 10 minutes, at 4 °C using a GSA rotor. The supernatant was decanted and the remaining portion was removed off cell-pellets using a micropipette. The cell pellets were placed on ice before being resuspended in ice-cold ATCase extraction buffer, pH= 8.5 (25 mL of 1M Tris-Cl, pH=8.5; 1mL of 1M BME; 0.01mL of 1M ZnSO₄; 0.4 mL of 0.5M EDTA, pH=8.0 brought to 1/2L of deionized water or ddH₂O). The weight of the resulting cell pellets was determined by first weighing empty centrifuge bottles (weight A), then bottles with pellets (weight B), finally subtracting weight A from weight B which amounted to total weight of cell pellet of 35.30 grams. Extraction buffer total volume is recommended to be twice the volume of cell pellet or 71.08 mL. After complete resuspension of cell pellets in the buffer, the cell-liquid was pooled into two 500 mL centrifuge bottles and cooled on ice for 1 hour. Cells were broken in a French pressure cell at 1,000-1200 pounds per square inch while maintained at 4 °C throughout the procedure. The resulting crude protein extract was collected into 30 mL Corex centrifuge tubes, chilled on ice. Unbroken cells and cell debris were removed by centrifugation at 8300 g for 10 minutes,

at 4 °C. The resulting supernatant, was transferred into a fresh centrifuge tube and respun for another 30 min at 8300 g, at 4°C.

The clarified rosy-pink supernatant was collected and stored on ice. 100 µL of it was saved for ATCase assay and protein assay.

METHODOLOGY USED IN ATCase ENZYME PURIFICATION

3.1. Streptomycin sulfate cut

Enzyme purification methodology used modifications of the Gerhart and Holoubek method (1967) which included a streptomycin sulfate cut, followed by a heat step and ammonium sulfate cut.

In the streptomycin sulfate cut, a 10 % (v/v) streptomycin sulfate solution was prechilled on ice. A 10 mL volume of it was added to a 50 mL conical tube containing 40 mL of the crude protein extract, resulting in a final concentration of streptomycin sulfate to be 2%(v/v)(final volume 50 mL). The protein extract/streptomycin mix was placed on ice in a prechilled pyrex baking dish or other glass dish, and completely covered with more ice. The dish was placed on a rotary platform at room temperature and mixed for 15 minutes at 50 rpm speed. The solution was then centrifuged at 8300 g for 10 minutes at 4 °C, then decanted the clarified rosy-pink supernatant from pellet into a prechilled 30 mL Corex tube. Resulting volume of supernatant was about 50 mL. A volume of 100 µL of supernatant and pellet were saved for ATCase activity and protein assay.

3.2. Heat step

The supernatant from the streptomycin sulfate cut was placed in a 60 °C water bath for 5 minutes, until the mixture showed white flocculent appearance. Then it was centrifuged at 8300 g for 10 minutes at 4 °C. The yellow-clear supernatant was decanted into a prechilled 50 ml conical tube and placed on ice. The 45 mL- volume of resulting supernatant was kept in ice until the salting out step. About 100 µL of supernatant and pellet were saved for ATCase activity/protein quantitation.

3.3. Ammonium sulfate cut

Salting out with a ammonium sulphate was performed using 390 g of solid salt per liter of solution to achieve final % saturation of 62. Scaling up for a solution volume of 45 mL, the amount of ammonium sulfate required was 17.55 g. This volume was divided into four portions, each in the amount of 4.388 g and placed into separate weighing boats. The experiment set up consisted of a glass dish of 1L capacity, filled with ice. A 150 mL capacity prechilled beaker, with a stirbar was placed into the glass dish, then the beaker was filled with the 45 mL clear protein extract (supernatant). The beaker was surrounded with more ice until half the dish-height. The stirbar was set at the lowest revolutions to avoid the protein extract from foaming while mixing (to prevent a too rapid protein precipitation and oxygenation). The first portion of the ammonium sulfate salt was added, stirred for 20 minutes on ice at RT or in a cold room until complete salt dissolution. The following salt portions were added every 20 minutes until all salt was used, for a total of 80 minutes. After completion of the last cut, additional 20 minutes were allowed of stirring on ice for equilibrium to be reached between aggregated proteins and dissolved

ones. The protein solution now appeared cloudy-yellow and its final volume was 56 mL. The solution was then transferred to two prechilled 30 mL Corex centrifuge tubes and spun at 8300 g for 10 minutes at 4 °C. The resulting supernatant (volume 46 mL) was decanted into a prechilled 50 mL tube and kept on ice. The pellet, which should include among others the salted out and precipitated protein of interest, ATCase, had approximate volume of 10 mL. The pellet was resuspended in 4-5 mL of prechilled ATCase extraction buffer, pH= 8.5 and kept on ice. 100 µL of supernatant was saved.

3.4. Microplate ATCase activity assay

The results of the sequential preliminary purification steps of the crude protein extract, were assayed for the presence of the protein of interest, ATCase, using the Microplate ATCase assay (modification of Prescott.& Jones, 1969, method).

The Microplate assay was performed by using a 96-well ELISA Microplate dish. The wells were marked with sample names to be tested, respectively: crude protein extract, streptomycin sulfate cut supernatant and precipitate, 60 °C heat step supernatant and precipitate, ammonium sulfate cut supernatant, ammonium sulfate cut precipitate, dialysis and two controls. Into each of the sample and control wells, 78 µL of ddH₂O was placed, followed by 4 µL of Tri- buffer, pH= 9.5 (50 mM MES or (2-(N-morpholino)ethanesulfonic acid), 100 mM diethanolamine, 51mM N-ethylmorpholine), then by 5µL of 20 X solution of aspartic acid (200 mM aspartate). Subsequently, 3µL of sample were placed into their respective wells, excluding the control wells. Sample volume was substituted in the control wells using deionized water. Lastly, 10 µL of

carbamylphosphate solution (100 mM solution or 0.04 g powder in 5 mL of ddH₂O prepared fresh before use or stored at -20 °C) was added into each well. The plate was tapped lightly against the benchtop to allow for the mixing of all ingredients within each well. The microplate dish was covered with a lid and incubated at 30 °C for 20 minutes to allow the ATCase to react with its two substrates, the aspartic acid and carbamylphosphate. Following incubation, the indicator solution was freshly prepared consisting of 2:1 ratio by volume of antypyrine and monoxime solutions. To each well then was added 100 µL of this mix, the microplate covered with a lid and incubated in light on a heating platform set at 60 to 65 °C, for one to two hours. The ATCase enzyme activity is manifested by the development of yellow color when the by-product of the enzymatic reaction reacts with the color indicators. The varying degrees of color intensity, corresponding to varying amounts of enzyme present, are then measured spectrophotometrically as absorbance at 450 nm values.

3.5. Dialysis

The precipitated protein extract from the ammonium sulfate cut was kept at 4 °C. Cellulose ester dialysis membrane was used, with Molecular Weight Cut Off (MWCO) of 15,000 Da (Spectra/Por[®]). The membrane tubing was cut into 5x 10 cm-long strips of 10 mm diameter, and soaked in deionized water for 30 minutes to remove the preservative. The tubes were clamped at one end and each filled with 300 µL (0.3 mL) of the concentrated protein extract. They were then closed at the other end and placed in prechilled 1L of ATCase buffer, pH=8.5 in a 4 L container (ATCase buffer: 0.05 M Tris-

Cl, pH=8.5; 0.002 M BME; 0.02 M ZnSO₄; 0.0002 M EDTA, pH=8.0 in ddH₂O). Dialysis was conducted at 4 °C with constant steering for 2 hours, then continued overnight in 2 L of fresh buffer. The total volume of concentrated protein extract before dialysis amounted to 1.5 mL, after dialysis the total volume was 23 mL. The dialysed protein extract was kept at 4 °C and used for subsequent purification.

2.6. Column chromatography using the GradiFrac System with HiLoad Pump P-50 and UV-1_Monitor from Pharmacia Biotech.

The following column order, as outlined below, is generally applied when performing routine protein purification, however the best results in this project have been achieved when the column order has been: ion-exchange, gel-filtration (size exclusion) and hydrophobic interaction chromatography, as described in Chapter 4.

3.6.1 Gel-Filtration chromatography or Size-Exclusion using Superdex 200 HiLoad (Sephacryl S-300) column from Pharmacia Biotech. (XK 16/60), fractionation range for globular proteins from 10,000 to 1,500,000 Da .

The column was initially washed with degassed ddH₂O at 1mL/minute flow rate for one hour and 10 minutes, at room temperature (25 °C) to eliminate preservative. Subsequently it was charged with ATCase buffer, pH=8.5 at the same flow rate for one hour and 30 minutes at 25 °C. The entire volume of dialysed protein sample was passed through a 1µm-pore size filter, to remove any impurities before applying it onto the chromatographic column. A 2.5 to 3 mL total volume of dialysed protein sample was placed onto the column, preceded by 1mL of Blue Dextran (2,000,000 Da). The sample

was run at a flow rate of 0.5 mL/minute, with an aliquot of 1mL, and total number of collection tubes being 70. Subsequently a Microplate ATCase assay was performed on tubes following Blue Dextran (V_0 - column volume). The sample tubes having the highest activity range were chosen and concentrated using the Milipore Ultra Free[®]-4 centrifugal filter unit, with Nominal MW Limit of 30,000 Da for 10 minutes at 4 °C. Three to four mL total of aliquot from the column run was concentrated 100 times, giving a final concentrate volume of 300-400 μ L. The entire dialysed partially purified protein extract was used and fractionated on the S-200 (or S-300) column, with 2.5 mL of sample per column run, for a total of 10 column runs or until the entire dialysed volume was used up. The resultant pooled fractions exhibiting highest ATCase activity by the ATCase Microplate assay, were used subsequently to be further purified.

3.6.2. Ion Exchange chromatography using Q Sepharose High Performance column from Pharmacia Biotech.(XK 26/10), exclusion limit for globular proteins of 4,000,000 Da.

The column was initially washed with degassed ddH₂O at 2 mL/minute flow rate for 30 minutes at room temperature to eliminate the preservative. Subsequently it was charged with degassed 1M NaCl ATCase buffer, pH=8.5 at a flow rate of 1mL/minute for 30 minutes then decharged with ATCase buffer, pH=8.5 for 30 minutes at the same flow rate. The sample was loaded with volume ranging from 0.5 to 1.0 mL. A buffer gradient was created from 0% NaCl salt to 100% NaCl salt, with a flow rate of 1mL/minute and 1mL aliquot fraction volume. A total of 60 tubes were collected and assayed for ATCase activity using the Microplate method. Tubes exhibiting the highest activity were chosen,

pooled and concentrated using the 30K Milipore filter unit for 10 minutes at 4 °C. This material was then used to be further purified.

3.6.3 Hydrophobic Interaction chromatography using the Phenyl Sepharose 6 Fast Flow (low sub) from Pharmacia Biotech (HiTrap HIC Test Kit, 1mL column).

The column was initially washed with degassed ddH₂O at flow rate 1mL/min for 30 minutes at room temperature to eliminate a preservative. Subsequently it was charged with a salt buffer of 0.4 mM of Ammonium sulfate salt in ATCase buffer, pH=8.5 at a flow rate of 1mL/minute for 30 minutes, decharged with a 0.2 mM Ammonium sulphate ATCase buffer, pH=8.5 at a flow rate of 1mL/minute for 30 minutes and charged again with the high-salt buffer used iniatially, at the same flow rate. Approximately 0.5 to 1mL of concentrated protein material from the previous column was used and adjusted to 0.4 mM salt concentration, then loaded onto column. The column was run at 0.5 mL/minute flow rate, 0.5 mL aliquot collected/tube, using a salt gradient from 0.4 mM ammonium sulphate to 0.2 mM ammonium sulphate in ATCase buffer, pH=8.5. Seventy tubes were collected and assayed for ATCase activity using the microplate assay. Tubes exhibiting the highest activity were chosen, pooled and concentrated in 30K Milipore filter unit for 15 minutes at 4 °C. The purified protein material was stored at 4 °C for further study.

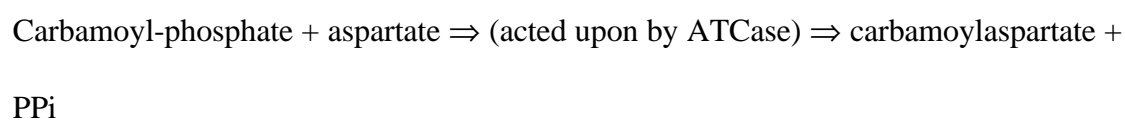
4. Protein Gel Electrophoresis

4.1 Activity gel

A 4%-8% non-denaturing gradient polyacrylamide gel (PAGE) was used. Linear slab gel Mini Vertical gel system was assembled, with .75 mm spacers between the glass plates.

To prepare an 8% (v/v) separating gel, 2 mL of Acrylamide/Bis mix solution in the ratio of 29:1 (BIORAD[®] N,N'-methylene-bis-acrylamide), 1.25 mL of 4x Separating gel buffer (1.5 M Tris-Cl, pH=8.8; and ddH₂O), 1.75 mL ddH₂O, 25 µL of 10% (v/v) ammonium persulfate were mixed together and degassed using a vacuum pump. Before loading the gel, 5 µL of TEMED was added to the solution, for a total of 5 mL. The separating gel was quickly poured between two glass plates using a Pasteur pipette to 1.5 cm from the top of the smaller glass plate. The gel was overlaid with sec-butanol and allowed to set for two hours at room temperature. Before casting the stacking gel, the butanol was poured off and the top of the gel rinsed several times with ddH₂O. To prepare a 4% (v/v) stacking gel, the solution comprising of 0.270 mL of Acrylamide/Bis mix solution, 0.4 mL of 4x stacking gel buffer (0.5M Tris -Cl, pH=6.8 and ddH₂O), 0.920 mL of ddH₂O, 15 µL of 10% (w/v) ammonium persulfate solution was mixed together, degassed as above. Before pouring, 10 µL of TEMED was added to start polymerization. After overlaying the separating gel, a comb was inserted between the plates until bottom of teeth reach the top of front plate. The stacking gel was allowed to polymerise for 30 minutes at room temperature. A 1x electrophoresis running buffer was prepared composed of 25 mM of Tris base (3.03 g/L), 192 mM of Glycine (14.4 g/L) in one liter of ddH₂O. After completion of polymerization, the comb was carefully removed and the gel assembly was placed in the electrophoresis tank and buffer poured into the gel chamber to submerge the wells created by the comb. Any loose acrylamide was washed off from the wells by using a squirt of buffer from a 10 mL syringe. The tank was filled with remaining buffer and any

air bubbles between the plates and gel chamber were removed using a good squirt from the syringe. Markers used were crude protein extracts from *Escherichia coli* wild type strain, and *Pseudomonas aeruginosa* wild type strain respectively. Samples included crude protein extracts from *Moraxella (Branhamella)catarrhalis* 25240 strain (strain used in this study) and for comparison *Moraxella osloensis* wild type strain and *Moraxella bovis* wild type strain, respectively. 10 μ L of each marker and 10 μ L of each sample were mixed with 2.5 μ L of 5x sample buffer (60 mM Tris-Cl pH=6.8, 25% (v/v) glycerol, 14.4 mM 2-mercaptoethanol, 0.1% (w/v) bromophenol blue, 2.9 mL ddH₂O). Marker and samples were each loaded into separate wells using a 25 mL Hamilton syringe. The gel was electrophoresed at room temperature at 100 V, 300 mA maximum for 1 hour and twenty minutes or until the tracking dye, bromphenol blue reached 5 mm from the bottom of the gel. At the end of the run, the gel was taken out from the gel asseby, washed at least three times in ddH₂O and used in the ATCase activity stain. The gel was placed in a pyrex dish and equilibrated for 5 minutes in 250 mL ice cold 50 mM histidine buffer, pH=7.0 (2.62 g of L-Histidine in 250 mL of ddH₂O). Subsequently, 5 mL of ice cold 1.0 M aspartate and 10 mL 0.1M carbamoylphosphate, respectively, were added to the gel/buffer bath and mixed gently on a shaking platform for 10 minutes (0.78 g of aspartate in 5 mL ddH₂O, 0.153 g carbamoylphosphate in 10 mL ddH₂O). The gel was then rinsed three times with ice-cold ddH₂O to remove reactants according to the following reaction:



The orthophosphate trapped in the gel (PPi) will form a white precipitate with lead, water insoluble lead phosphate, upon addition of ice cold 3 mM lead nitrate in ice cold 50 mM L-histidine buffer, pH=7.0 to the gel, and incubating 10 minutes (0.25 g lead nitrate in 250 mL of histidine buffer). The lead nitrate was removed by rinsing the gel three times with ice cold ddH₂O. The gel was stored overnight at 4 °C in ddH₂O, the dish being covered with Saran Wrap to eliminate evaporation. The activity gel for ATCase revealed the presence and approximate molecular weight of the functional holoenzyme, as compared with the markers. The white lead phosphate precipitate's resolution could be further enhanced by converting it to dark lead sulfide precipitate by submerging the gel in 300 mL solution of 1% (w/v) sodium sulfide. After 5 minutes, the sulfide solution is removed by rinsing the gel with tap water for about 30 minutes to eliminate background staining. To remove additional background, the gel could be further destained with 0.7% (v/v) nitric acid.

4.2 A 4-10% gradient denaturing polyacrylamide gel was used (SDS-PAGE).

To prepare a 10% separating gel for a total volume of 5 mL, 1.75 mL of ddH₂O was mixed with 2 mL of solution A (Acrylamide/Bis mix solution in the ratio of 29:1), then with 1.25 mL of solution B (4x separating gel buffer, pH=8.8 containing 0.1% (w/v) SDS), and 25µL 10% (w/v) ammonium persulfate. After degassing, 2.5µL of TEMED was added just before loading the gel between the gel plates. The electrophoresis 1x running buffer was freshly prepared by mixing 3.03 g of Tris (25 mM), 14.4 g glycine (192 mM) and 1.0 g 0.1%SDS (w/v) in a total volume of one liter of ddH₂O. Samples were

prepared by mixing 10-15 μL with 2.5-3.0 μL 5x sample buffer (containing SDS) in individual 0.5 mL Eppendorf tubes. 10 μL of marker (Mid Range Marker from Biolabs) was also prepared and mixed with 0.5 μL of a reducing agent such as DDT (Dithiothreitol). The protein marker and samples were heated to 95-100 $^{\circ}\text{C}$ in water bath for 3 minutes before loaded onto the gel, to unfold the proteins. The samples were loaded onto the gel (4%-10% stacking/separating gel SDS-PAGE) prepared as for non-denaturing PAGE and run at 25 $^{\circ}\text{C}$ at 100 V for 1hour and 20 minutes. After electrophoresis, the gel was rinsed in ddH₂O to remove the buffer and stained in Coomassie Blue R-250 solution for 20 minutes on a rotary shaker at 25 $^{\circ}\text{C}$. The gel was subsequently destained in Coomassie Gel destain solution with 2-3 changes of the destaining solution to remove background stain or was left to destain overnight, if necessary. The gel was stored in ddH₂O before drying.

4.3 Electroblothing from SDS-PAGE gel onto Polyvinylidene difluoride (PVDF) membrane.

The concentrated and purified ATCase from the hydrophobic column was run on a 4%-8% SDS-PAGE gel in Tris-glycine buffer to separate the holoenzyme into its corresponding subunits. The protein fragments were electroblotted and immobilized onto a Polyvinylidene difluoride (PVDF, capacity of $\sim 150 \mu\text{g protein/ cm}^2$) membrane in preparation for N-terminal amino acid sequence determination. After gel electrophoresis at 100 V for one hour and 20 minutes, the gel was rinsed in ddH₂O three times to remove residual buffer and kept in water at 4 $^{\circ}\text{C}$. Seven pieces of 3MM Whatman paper were cut

the size of the gel. The PVDF membrane was cut the size necessary to cover the area on the gel where the protein fragments of interest have migrated. A transfer buffer was prepared (Tris-glycine with 15% (v/v) Methanol in one liter of ddH₂O) and cooled to 4 °C. Two Scotch-Brite pads and the 7 Whatman pieces of paper were soaked in the transfer buffer for 15-30 minutes. Before use, the Scotch pads were squeezed out to remove air pockets and one of them placed on the inner surface of the grey-colored panel of the gel holder. Four buffer soaked 3MM Whatman filter papers were placed onto the pad, one at a time, each time removing any trapped air bubbles by rolling then out with a glass rod or pipette. The paper surfaces were kept moist with transfer buffer. The gel was placed onto the surface of the paper, face-up, prewetted with transfer buffer. Again air bubbles were smoothed out with a glass rod. The prewetted PVDF membrane was placed carefully over the gel, flooded with transfer buffer and air pockets smoothed out as before. When preparing the PVDF membrane, it was immersed in 100% (v/v) Methanol for a few seconds, until translucent. Then it was quickly placed into transfer buffer and equilibrated at 25 °C for 2-3 minutes (when completely equilibrated, the membrane will float onto the surface of buffer). At this point the membrane is ready to bind proteins. The moist membrane was quickly covered with the remaining three sheets of 3MM Whatman paper (prewetted in transfer buffer), one at a time, all air pockets smoothed out as above to assure complete surface to surface contact. The resulting stack was finally covered with a second saturated Scotch Brite pad and the gel holder/cassette was closed. The transblot cell was half filled with ice cold transfer buffer and the gel cassette was placed into it, with the grey-colored panel facing the cathode. The cooling container (kept at -80 °C) was

inserted next to the cassette to prevent the system from overheating during electrophoresis. The gel tank was filled to its full holding capacity with the remaining transfer buffer and the assembly was placed onto a stirring platform. A stirring bar was placed into the transblot cell to allow even distribution of cool buffer over the gel cassette during electrophoresis. The gel was electroblotted at 25 °C or in the cooldroom at 100 V for one hour and 20 minutes (amperes were not allowed to exceed 300 mA). After transfer, the membrane was removed from the gel cassette and a corner cut with a clean razor to mark orientation. The membrane was briefly rinsed in ddH₂O, then soaked in 100% (v/v) methanol for a few seconds before being stained in the Coomassie Stain R-250 solution for one minute with shaking. The membrane was subsequently destained in 50% (v/v) methanol, with a change of destaining solution every 20 minutes or so. When most of the background stain was removed the membrane should present dark contrasting bands against a clear background. The membrane was finally rinsed in ddH₂O and allowed to dry completely on a piece of paper towel or Kim-Wipe for 30-60 minutes to increase contrast of the bands. The dried membrane was sealed in a plastic baggy and stored at 4 °C before being shipped for sequence analysis. The original gel was retained, in case not all protein has been transferred onto membrane.

CHAPTER 4

RESULTS

FIGURE. 4.A. SIZE EXCLUSION (GEL FILTRATION) CHROMATOGRAPHY. Sephacryl S-300 column. Sample material (Batch C) from dialysis applied to the column. Elution volume at peak ATCase activity is 45mL.

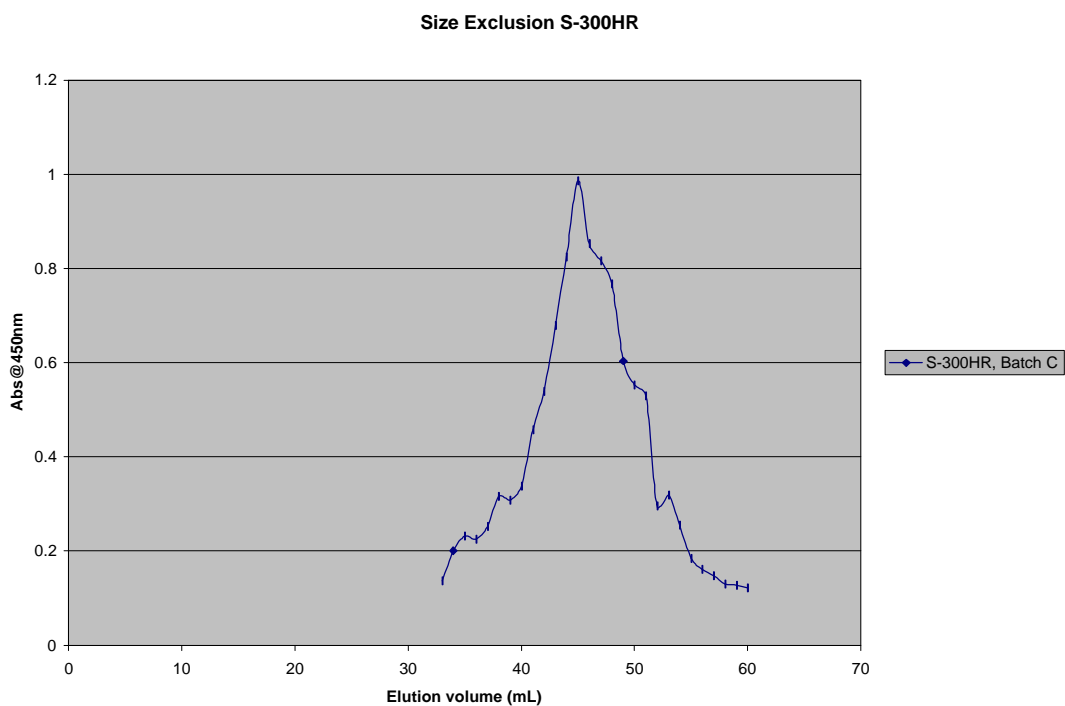


FIGURE 4.B. IONIC INTERACTION CHROMATOGRAPHY.
Anion Q Sepharose HP. Sample material (Batch2) applied from size-exclusion column.
Elution volume at peak ATCase activity was 23mL.

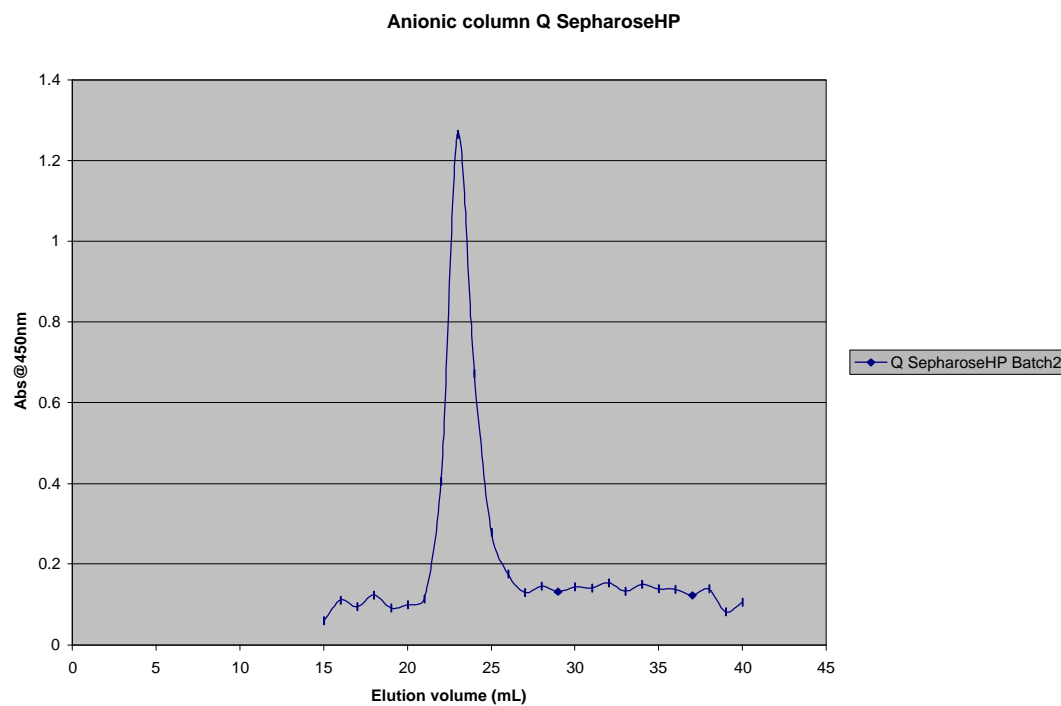


FIGURE 4.C. HYDROPHOBIC INTERACTION CHROMATOGRAPHY.

Phenyl Sepharose 6 FF(low sub) with volume capacity of 1mL. Sample material applied from anionic column. Peak ATCase activity shows at ~ 200 mMol ammonium sulfate concentration.

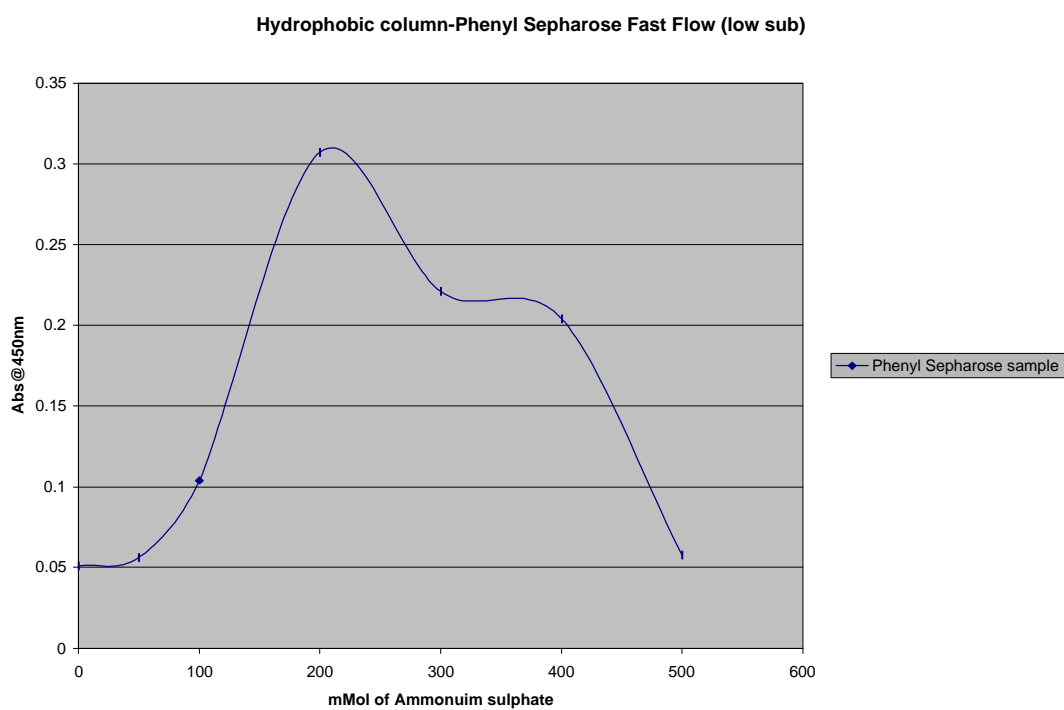


FIGURE 4.D. BRADFORD PROTEIN ASSAY.

STD used: Serum Gamma Globulin. Sample values range correspond from crude protein extract (value 0.189) to HIC column (value 0.229).

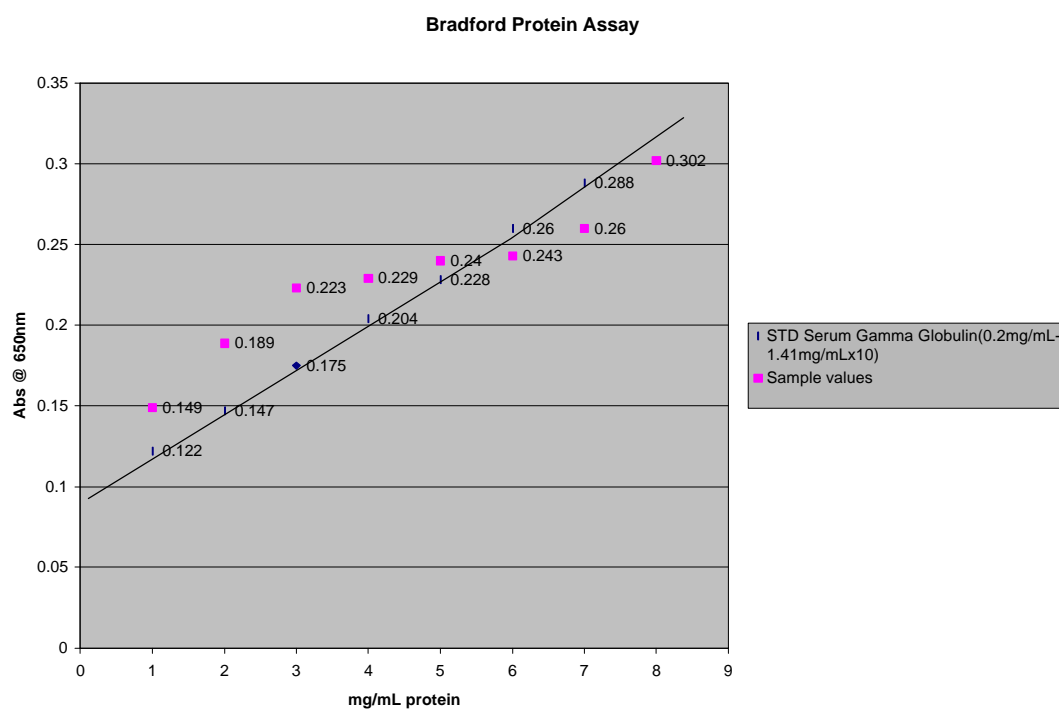


FIGURE 4.E. CARBAMOYL ASPARTATE (CAA) CURVE.

Sample values range from crude protein extract (value 0.289) to HIC column (value 0.345).

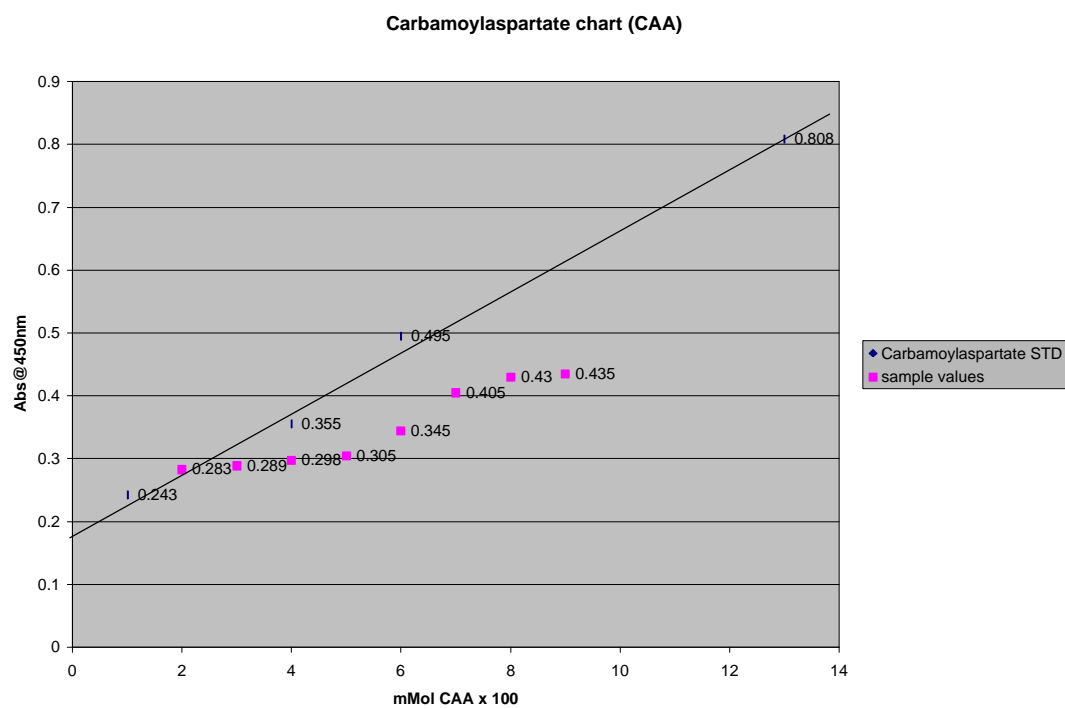


TABLE 1. PURIFICATION ANALYSIS OF ATCASE FROM
Moraxella(Branhamella)catarrhalis 25240

Sample	Volume (mL)	Abs@ 450nm	Concentration of protein ($\mu\text{g}/\mu\text{L}$)	Total activity ($\mu\text{moles}/\text{min}$)	Specific activity ($\mu\text{moles}/\text{min}/\mu\text{g}$)	% Yield	Fold purification
Crude Protein Extract	40.0	1.289	28.0	2.4×10^8	214.3	100	-
Streptomycin sulfate cut supnt.	50.0	0.886	13.5	1.033×10^8	153.0	43.04	0.714
Heat treatment 60°C supnt.	45.0	0.933	>14.1	1.21×10^8	190.3	50.3	0.88
Ammonium sulfate cut ppt.	5.0	2.978	142	1.375×10^8	19.5	5.73	9.036
Dialysed protein extract.	23.0	0.435	3.3	1.476×10^7	194.44	6.15	0.907
S-300 size excl. column	23.0	1.344	31.5	1.45×10^8	201.1	60.6	93.81
Anionic column	23.0	1.265	27.0	1.303×10^8	209.87	54.3	98.0
HIC column	1.0	0.482	4.42	7.8×10^5	177.2	0.326	82.7

FIGURE 4.F. 4%-8% POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)
ACTIVITY GEL.

Markers: Lane 1-*Escherichia coli* protein standard of 310 kDa and 100 kDa respectively.

Lane 2- *Pseudomonas aeruginosa* protein standard of 480 kDa; 10 μ L per lane, each.

Samples: Lane 3- *Moraxella(Branhamella)catarrhalis* 25240; Lane 4- *Moraxella bovis*;

Lane 5- *Moraxella osloensis*; 10 μ L per lane of each.

The three samples of *Moraxellae* spp. co-run with the *Pseudomonas aeruginosa* marker.

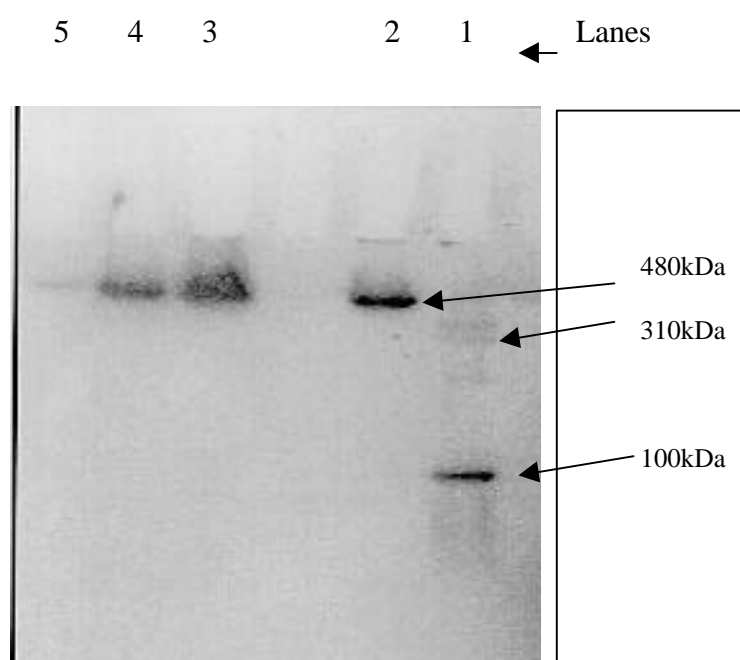


FIGURE 4.G. 4%-10% SDS-PAGE GEL. Anionic column chromatography.

Molecular Weight Marker: Promega Mid-Range Lane 1:- 97.4 kDa, 66.2 kDa, 55.0 kDa, 42.7 kDa, 40.0 kDa, 31.0 kDa, 21.5 kDa and 14.3 kDa; 10 μ l per lane.

Sample lanes: Exhibit strongest ATCase activity: Lane 3 – sample #.15; Lane 4- sample #.16; and Lane 5- sample #. 17; 10 μ l per lane.

Lanes: 1 2 3 4 5 6 7 8 9 10

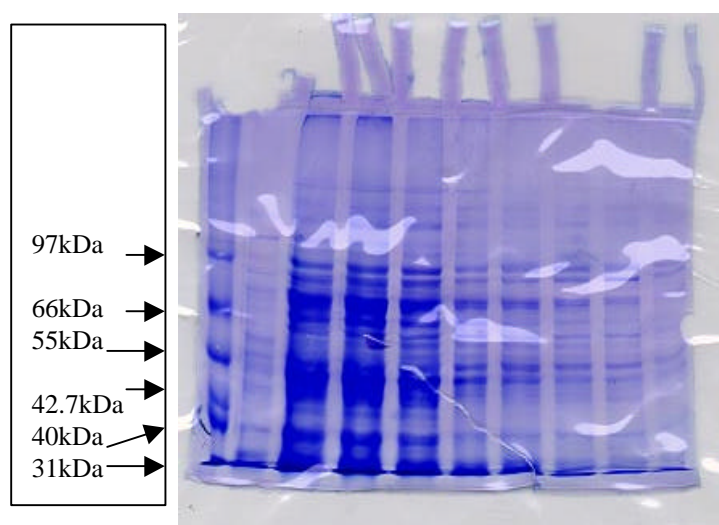


FIGURE 4.H. 4%-10% SDS-PAGE GEL. Gel Filtration chromatography

Molecular Weight Marker: Promega Mid Range (Lane 1): 97.4 kDa; 66.2 kDa; 55 kDa; 42.7 kDa; 40 kDa; 31 kDa; 10 μ l per lane.

Sample lanes: Highest ATCase activity seen in Lane 3 and 4, correspond to 34mL and 35mL sample volume, respectively; 10 μ l per lane.

Lanes: 1 2 3 4 5 6 7 8

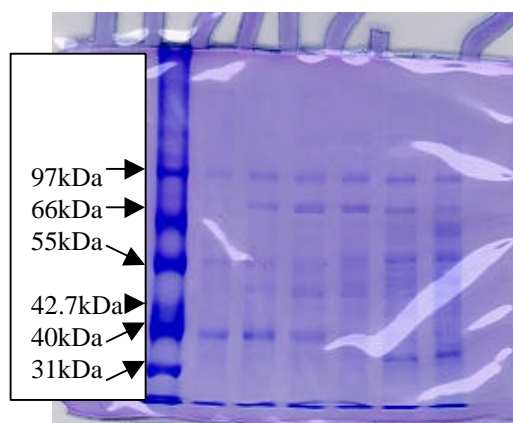


FIGURE 4.I.1. 4%-10% SDS-PAGE GEL. Hydrophobic Interaction chromatography.

Molecular Weight Marker: Promega Mid Range (Lane 1): 97.4 kDa; 66.2 kDa; 55 kDa; 42.7 kDa; 40 kDa and 31kDa; 10 μ l per lane.

Sample lanes: Lane 2 – 700 mM ammonium sulfate; Lane 3- 500 mM; Lane 4- 200 mM; Lane 5- 100 mM; Lane 6- 50mM and Lane 7- no salt ATCase buffer, pH=8.0; 5 μ l per lane.

Figure 4.I.1.

Lanes: 6 5 4 3 2 1

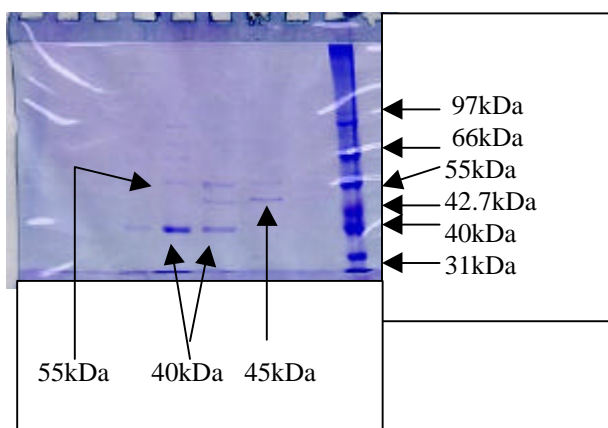
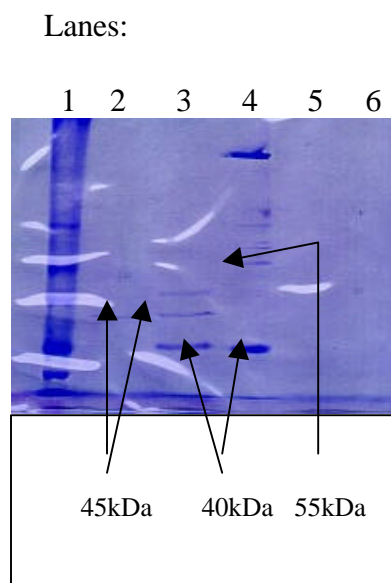


FIGURE 4.I.2. 4-10% SDS-PAGE GEL. Hydrophobic Interaction chromatography.

Molecular Weight Marker: Promega Mid Range Lane1: 97.4 kDa; 66.2 kDa; 55 kDa; 42.7 kDa; 40 kDa and 31kDa; 10 μ l per lane.

Sample lanes: Lane 2- 700 mM ammonium sulfate; Lane 3- 200 mM and Lane 4- 100 mM ammonium sulfate in ATCase buffer, pH=8.0; 5 μ L per lane.

Figure 4.I.2.



CHAPTER 5

CHAPTER 5

DISCUSSION

Preliminary work on the ATCase from *Moraxella (Branhamella) catarrhalis* performed by the laboratory of Dr. Mark Shanley (Fowler, 1998), and achieved the partial purification of this enzyme. As the final steps of the purification process, DEAE cellulose anion exchange column and Sephacryl S-300 HR size exclusion column chromatography methodology were used.

In this project, a complete purification of the ATCase enzyme from *M.(B).catarrhalis* was attempted sufficient to allow a N-terminal amino acid sequence determination.

An activity gel has been prepared using a 4%-10% PAGE gel (non-denaturing conditions). Samples of *M.(B.) catarrhalis* 25240 strain were used from the size-exclusion column chromatography. Additional samples for comparison were used from other *Moraxella* genera, notably *M. bovis* and *M. osloensis*. The latter two species were grown separately in BHI growth media (*M. bovis* medium had to be enriched with FITC). Both were grown at 37 °C incubator shaker in a 50 mL volume using 250 mL flasks. The cells were pelleted at 6000 g for 10 minutes and resuspended in ice-cold ATCase breaking buffer, pH=8.5. Each cell suspension (about 2mL) was sonicated while kept on a ethanol/ice slurry bath, for a total of 8 minutes with 1minute intervals, in pulse mode.

They were subsequently centrifuged as above to pellet cell debris. The supernatant was used in the activity gel. The activity gel was loaded with marker-species:- *Escherichia coli* 310 kDa and 100 kDa, *Pseudomonas aeruginosa* 480 kDa. An aliquot of 15 μ L of each sample and markers were mixed with 3 μ L of 5x loading dye. The gel was loaded in the following order: *E.coli* marker, *P.aeruginosa* marker, *M.(B.) catarrhalis* 25240, *M. bovis* and *M. osloensis*. The gel was electrophoresed in Tris-Glycine buffer at 50 V for 5 minutes then 100 V for 1hour and 25 minutes. The gel was treated for activity test as described in section 3.1.

The activity gel (Fig.4., pg. 49) reveals that all the samples, including *M.(B.) catarrhalis* co-run with *P. aeruginosa* marker as single bands, thus demonstrating that the molecular weight of the *M.(B.) catarrhalis* 25240 ATCase holoenzyme is equal to or slightly larger than 480 kDa of the *P. aeruginosa* ATCase. *M. bovis* and *M. osloensis*, belonging to the same genus as *M.(B.) catarrhalis*, prove to possess the same size ATCases. The molecular weight of ATCase holoenzyme for *M.(B.) catarrhalis* has been determined in the previous study (Fowler, 1998) using the S-300HR Sephacryl chromatography column, and by extrapolating from the standard curve using the V_e/V_o vs. log MW, it was found to lie between 480-520 kDa. As previously described, numerous studies have established the molecular mass of ATCases in various organisms, such as the *Pseudomonas* species (Adair & Jones, 1972). The purification sequel that followed for *M.(B.) catarrhalis* 25240 ATCase applied the standard method in protein purification, which included: obtaining a crude, cell-free protein extract; streptomycin sulphate cut;

heat step; ammonium sulphate cut; dialysis; column chromatography such as size-exclusion, anionic and hydrophobic interaction (Rosenberg, 1996).

At each purification step it is necessary to perform quantitation analysis of the studied protein so that one can monitor its concentration, assess removal of unwanted proteins and determine its specific activity (if it has enzymatic properties) and purity. The presence of the enzyme ATCase at each purification step has been determined by applying an activity test, specific for that enzyme. In the ATCase microplate assay, the ATCase is allowed to react with two substrates: aspartate and carbamoylphosphate at concentrations of 200 mM and 100 mM, respectively. The enzymatic reaction is maintained at 30 °C for a time of 20 minutes in the presence of 3 μ L of sample per 87 μ L total reaction volume. The Tri-buffer used in the reaction well is composed of 50 mM MES, 100 mM diethanolamine and 51 mM N-ethylmorpholine at a pH of 9.5. The resulting product of the enzymatic reaction, carbamoylaspartate (CAA), is detected by reacting with a stop solution (2:1 parts of antipyrine: monoxime) to give a yellow color, after being kept on a 60 °C heating plate in light for about 1.5-2 hours. This colorimetric reaction is read using a spectrophotometer at 450 nm to determine the different intensities of the color, in each sample well. Two control blank wells that do not contain any sample, are also included in with the sample wells for zeroing reading by the spectrophotometer. Another buffer, Tris-Cl buffer at pH of 9.5, was found to work equally good as the Tri-buffer in this test.

Table #.1, (Chapter 4, pg. 48) summarizes the results of all these steps.

The optical density for each sample read at 450 nm wavelength, corresponded to the amount of carbamoylaspartate (CAA) formed as a result of the enzymatic reaction (Prescott, 1969). This value for each sample, in mM CAA, was deduced from a standard curve (Fig.4.E., pg. 47). The CAA value for each sample in mM was used to calculate the enzyme activity for each sample, in $\mu\text{moles}/\text{min}/\mu\text{L}$. Enzyme activity values were used to calculate the specific activity of ATCase, in $\mu\text{moles}/\text{min}/\mu\text{g}$ which estimates its activity per μg amount of protein in the sample. Protein concentrations of each sample were deduced from the modified Lowry Protein Assay STD curve (Fig. 4.D., pg. 46). As can be seen from Table #. 1, absorbance varies slightly in the preliminary two steps of the purification process as unwanted components are eliminated. Performing a streptomycin sulfate cut (2% w/v) eliminates preferentially ribosomal proteins from the crude protein extract mixture of proteins, which after centrifugation, are removed as a pellet from the whole extract. Heat treatment at 60 °C for 4-5 minutes, eliminates mostly DNA-bound proteins, which precipitate in a whitish and flocculent form. This particular property of ATCase, temperature stability, facilitates its purification, where heat sensitive proteins are selectively eliminated from the protein mixture while ATCase retains its enzymatic activity unchanged. The overall specific activity of the enzyme diminishes by 11.2% as compared with the crude extract on reaching the heat treatment step, but the degree of purification is improved by 19%. In order to preserve the physiological and morphological stability of a target protein during the course of the purification process which includes storage, high concentrations of salts are used. Typical salt used is ammonium sulfate, usually at

centrations exceeding 50%. High salt environment stabilizes enzymes and generally inhibit the action of proteases or protein-degrading enzymes (Rosenberg, 1996). Thus the next step in the purification process is an ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$) cut or “salting- out”, whence the solubility of the target protein is decreased in a high concentration of this salt and the protein precipitates out of solution. This not only enhances the purification of the target protein but also reduces the sample volume, as in dilute solutions enzymes rapidly lose their activity.

Previous studies on *M.(B). catarrhalis* ATCase (Fowler, 1998) has showed that the enzyme reaches saturation point at 25% (w/v) ammonium sulfate and precipitates out of solution at 60%-62% (w/v) ammonium sulfate concentration. The ammonium sulfate cut procedure is performed on ice to maintain enzymatic stability and decrease solubilities. This study has used 62% (w/v) ammonium sulfate salt concentration to precipitate ATCase out of solution thus placing this enzyme as moderately to highly hydrophobic. The final volume in which the pellet of salted out proteins was resuspended, was 5 mL of ATCase extraction buffer, pH of 8.5 thus reducing the volume of sample to 88.9%, while increasing the mass of protein by a factor of 10. This preserves the ATCase enzymatic activity for long periods of time while in storage at 4 °C. Before applying the protein onto chromatographic columns, aliquots of a total of 1.5 mL of salted out protein were dialysed against the buffer environment that was subsequently used in the columns. The dialysis process removes excess low molecular weight solutes and equilibrates the sample in the new buffer environment. The dialysis tubing used in the study was made of a cellulose ester semi-permeable membrane with a molecular weight cut-off (MWCO) of 15,000

which means that, molecules below that molecular weight limit can freely pass across both sides of the membrane and reach new equilibrium while molecules above that limit cannot cross the pores of the membrane and are thus retained inside the dialysis tubing.

The volume increase after dialysis of the sample under study, is from 1.5 mL to 23.0 mL accounting for the decrease in concentration of the protein per mL but a visible increase in specific activity by a factor of 10. This is due to the fact that the “salting –out” process during the ammonium sulfate cut dehydrates the microenvironment of the protein molecule. When proteins are in solution, the hydrophobic areas on the protein molecule are covered with a shell of water molecules. The water molecules within the shell are ordered to maximize hydrogen bonding but this ordering is energetically unfavorable. By gradually increasing the salt concentration during the “salting-out” process, water molecules are pulled off from the hydrophobic regions to associate with salt ions, in this case, the sulfate ions. This process reduces the amount of water molecules available to interact with the protein and exposes the hydrophobic patches which subsequently interact with hydrophobic patches on neighboring molecules, forcing them to become less soluble and aggregate as a precipitate. The increase in volume during dialysis, re-hydrates the hydrophobic areas on the molecule and thus makes it soluble again (Bradshaw, 1998; Rosenberg, 1996). The increase in specific activity after dialysis to almost the same value as before the ammonium sulfate cut (190.3 and 194.4 $\mu\text{moles}/\text{min}/\mu\text{g}$), considering the decrease in the amount of protein per μL (from >14.1 to $3.3 \mu\text{g}/\mu\text{L}$), is testimony that the “salting –out” process has preserved the enzyme’s function and has increased its purity by a factor of 2.8%.

60 Previous studies performed on ATCase from *M.(B). catarrhalis* (Fowler, 1998) have provided an estimate of the enzyme's molecular mass to be between 480-520 kDa by using gel-filtration chromatography column, Sephacryl S-300HR.

In the initial part of this project, our lab has used the following order to conduct the final steps of ATCase purification, namely: ionic-column, gel-filtration and hydrophobic interaction.

The ion-exchange chromatographic column used was Q Sepharose HP (Fig. 4.B., pg. 44 and Fig. 4.G., pg. 50). A total of 12 mL of dialysed sample was applied to the column, run at a flow rate of 2 mL per min and collection volume 2 mL per tube [1]. The ATCase buffer gradient ranged from ATCase buffer, pH=8.0 to 0.75 M NaCl ATCase buffer of the same pH. A total of 65 tubes were collected or 130 mL total volume. The ATCase activity test on the samples from each tube revealed highest activity range to be in tubes #.16-20, with the peak activity in tube #.17. This would roughly correspond to a column volume of 34 mL or NaCl concentration of 0.196 M.

Proteins and peptides possess either a net positive or negative charge that vary with pH, thus being positively charged at pH values below their isoelectric points (pI) and negatively charged at pH values above their pI. The mechanism of separation of proteins on the anion-exchange chromatography depends primarily on reversible, ionic or electrostatic interactions between proteins and salts in the mobile phase (buffer) and a positively charged ion-exchange group contained on the stationary phase (matrix), based on the pH-dependent charges (Bradshaw, 1996). Q Sepharose is a strong anion column where the positively charged group on the matrix is a quaternary ammonium group. The

mobile phase contain negatively charged counterions such as Cl^- , that compete with the negatively charged protein attached to the positive groups on the matrix, thus selectively displacing the protein as the concentration of the Cl^- ions in the buffer phase increase. Previous studies (Fowler, 1998) have revealed that the optimum pH for ATCase enzymatic activity is between 9.0 and 9.5, but its pI is not known thus it cannot be separated effectively by adjusting the pH to a precise value at which the enzyme would elute in pure form. Maintaining the buffer at a constant pH of 8.0, creates initially (beginning of gradient with no salt) a negatively charged environment so most of the positively charged amino acid side groups facing the hydrophilic external environment of the protein molecule will be neutralized. This would leave predominantly negatively charged and non-ionizable side groups exposed. Since with increasing gradient, relatively few Cl^- counterions in the buffer are required to displace the ATCase and other proteins co-eluting with it, (the ATCase elutes at 0.196 M NaCl, early in the gradient thus exhibiting a weak interaction with the matrix of the column), it has to harbor predominantly positive charges.

The subsequent column used was the gel-filtration chromatography, Superdex 200 HiLoad (Fig. 4.A., pg.43 and 4.H., pg.51), [1] with a bed volume of 320 mL, with a fractionation range of 10,000-600,000 Da and sample volume of up to 13 mL. Protein samples from the anionic column were collected from tubes #. 16-24 and pooled to a final volume of 18 mL then concentrated to a volume of 1.5 mL in a 30 MWCO concentrator. The column was charged with ATCase buffer, pH=8.0, then was preliminarily loaded with a 1mL of Blue Dextran, followed by 1mL of the sample. The column was run at a flow

rate of 0.5 mL per minute, with a fraction of elutate volume being 1.5 mL. A total of 56 tubes were collected amounting to a total volume of 84 mL. An ATCase activity test revealed activity range from tube #. 32-43 with the strongest activity in tubes #.33-35, and peak activity in tube #.34. This tube corresponds to a elution volume (V_e) of 51mL. This gel does not serve to determine the ATCase molecular mass, since this has been done already in a previous study (Fowler, 1998), but to serve as a purification tool. By comparing Fig. 4.H. and Fig.4.G., one can see that the anionic column chromatography has significantly reduced the number of contaminating proteins, especially in the high molecular mass range.

The last column used was the HiTrap HIC Test Kit Phenyl Sepahrose 6 FF (low sub) column (Figs.4.I.1., pg. 52 and I.2., pg. 53),[1]. Resolution in Hydrophobic Interaction Column (HIC) is based on the relative strength of interaction between the non-polar matrix of the column and the hydrophobic regions on the protein. Phenyl-group on the matrix increases protein retention times as the hydrophobicity of the functional groups on the protein increases. The weak hydrophobic interactions of the protein with the phenyl groups is achieved through the use of high ionic strength buffer of such a salt as ammonium sulfate. The principle of protein resolution is based on the “salting-out” of proteins onto the solid support, by exposing their hydrophobic areas. After binding to the matrix in the presence of high salt concentration, typically 1-3 M, the protein of interest is eluted with a descending linear salt gradient that allows the polypeptide to resolvate (with water), thereby causing association with the hydrophobic matrix to be thermodynamically unfavorable. Being back in solution, the protein elutes as a soluble entity. Ammonium

sulfate salt ((NH₄)₂SO₄) is most popular choice used in HIC column chromatography, because it has a high aqueous solubility (approx. 4 M at 25 °C) and has a relatively high surface tension (2.17 $\sigma \times 10^3$ dyn-g/cm-mol; usual range being lowest, 1.50 for KCl and highest, 3.12 for sodium citrate). An increase in the buffer (mobile phase) surface tension should lead to an increase in solute retention on the matrix (stationary phase) of the column, thus more hydrophobic proteins should require salts with higher surface tensions and of high concentration to maximize selectivity and resolvability, as the salt concentrations are decreased. The pH of the buffer also affects the protein's retention time for, in general, an increase in pH reduces hydrophobic interaction thereby decreasing retention time (Bradshaw, 1998). A pH of 8.0 would thus increase the retention time of the ATCase on the column.

This 1mL capacity column could accommodate up to 0.5 mL of sample [1]. Samples from the previous column chromatography, tubes #.33 and #.34, were pooled for a total of 3 mL. This sample was concentrated to a volume of about 0.5 mL using a 10 MWCO concentrator. Before loading the column, the sample was prepared by mixing 0.150 mL of it with 0.350 mL of 1M ammonium sulfate for a final concentration of 0.7 M salt for a total of 0.5 mL. A salt gradient was run ranging from 0.7 M ammonium sulfate in ATCase buffer, pH=8.0, 0.5 M salt, 0.2 M salt, 0.1 M salt, 0.05 M salt to buffer without salt. Volume of the aliquots was 1mL, flow rate approximately 1mL per minute. The aliquots were assayed for ATCase activity using the microplate assay. Highest activity was detected in the salt concentration range of 0.1-0.2 M, with peak activity being around 0.196-0.2 M ammonium sulfate concentration. The samples were concentrated in a 10

MWCO concentrator and a 5 μ L (mixed with 2 μ L of 5X loading buffer) aliquot of each were run on 4%-10% SDS-PAGE gel against Promega Midrange Protein marker, at 50 V for 5 minutes then at 100 V for 1hour and 25 minutes in Tris-glycine buffer.

The gel pictures depicted in Figs.4.I.1and 4.I.2. both demonstrate that in lane #.1 (0.7 M salt), there is a very faint band running at approximately 45-46 kDa, coresponding to very weak ATCase activity. In Fig. 4.I.1, lane #.2 (0.5 M salt) corresponds to weak ATCase activity and shows two bands, one running at 45-46 kDa and the other at approximately 55-56 kDa. The 45-46 kDa band becomes weaker in #.3 (0.2 M salt), whereas the 55-56 kDa band becomes stronger. A third band appears, corresponding to approximately 40.0 kDa. These three bands co-elute at an ammonium sulfate concentration of 0.2 M and very strong ATCase activity. However, lane #.4 (0.1M salt) shows the 45-46 kDa band dissapear completely while the 55-56 kDa band is very weak (the Fig. 4.I.2. shows no band coresponding to 55-56 kDa at 0.1M salt concentration) while the 40.0 kDa band has its highest intensity at the ammonium sulfate concentration of 0.1M and strong ATCase activity in both gels (Figs.4.I.1and 4.I.2). Several additional faint bands at molecular mass above 60 kDa also co-elute at 0.1M salt concentration in both gels, being contaminants. In gel Fig. 4.I.1., lane #.5 corresponding to 0.5 M salt, shows the 40 kDa band with weaker intensity (weak ATCase activity), the 55-56 kDa band dissapears, while the 55-56 kDa bands persist. The lane #.6 shows extremely weak 40.0 kDa band and contaminating bands, with coresponding weakest ATCase activity.

These results can be interpreted taking into consideration the following facts.

In Chapter 2, different classes of ATCases are discussed, discovered and described by various authors. As mentioned, the largest ATCases are found in the genus *Pseudomonas* and their holoenzymes and subunit composition discussed (Schurr, 1992 and 1995; Bergh, & Evans, 1993). Class A ATCases are large holoenzymes, with a molecular mass of about 480 kDa. Their catalytic trimers are made of a 34-kDa identical polypeptides, while the dimers are made of 45-kDa identical polypeptides of imprecise property.

Stoichiometrically, the structure of the holoenzyme consists of an association of six 34 kDa-polypeptides with six 45-kDa-polypeptides, a dodecamer or a twelve-subunit ensemble with a total mass of 474 kDa. Studies performed on *M.(B.) catarrhalis* ATCase holoenzyme (Fowler, 1998) and corroborated by this study, have shown that the ATCase molecular mass is identical or very close to *P. aeruginosa* ATCase mass, of about 480 kDa to 520 kDa range, being evidently larger than *Escherichia coli* ATCase. This would suggest placing the *Moraxella* ATCase within the Class A ATCases. The two subunit sizes have been suggested by the Fowler study to be approximately 35 kDa and 45 kDa, respectively, using samples from the size-exclusion chromatography. In our study, one can infer from the observations of HIC gels in Figs. 4.I.1 and 4.I.2., that the first bands to appear are the 45 kDa and 55 kDa at 0.5 M ammonium sulfate salt (weak ATCase activity), the 45 kDa being the strongest of the two. At the strongest range of ATCase activity, corresponding to ammonium sulfate salt concentration range of 0.1 to 0.2 M, the 55 kDa band becomes more prominent, the 40.0 kDa band appears while the 45 kDa band weakens. At the 0.1M salt, the 45k Da band disappears, the 55 kDa band is very weak and the 40 kDa band is the strongest. This would suggest the 40 kDa subunit having a catalytic

function. Also worthy of notice is the fact that at the salt range of strongest ATCase activity (0.1-0.2 M salt) and specifically at 0.2 M salt, three bands coelute: the 40 kDa, 45 kDa and 55 kDa band, respectively. Having in mind the suggested similarity of *M.(B.) catarrhalis* ATCase holoenzyme as a class to *Pseudomonas* spp., and thus in its subunit composition, the two gel samples from the HIC columns depicted in Figs.4.I.1. and I.2. suggest that in the *M.(B.) catarrhalis* 25240 ATCase, the two subunits masses are about 40.0 kDa and 45.0 kDa respectively, with the 40.0 kDa being probably the smaller catalytic subunit. This would make the total mass of the holoenzyme to be 510 kDa (a dodecamer or 6x 40 kDa + 6x 45 kDa), which is consistent with the results of the activity gel (Fig. 4.G.) where *M.(B.) catarrhalis* ATCase migrates at the same level as *P. aeruginosa* ATCase, 480 kDa. Whether the 55-56 kDa band can be included as a relevant component of this organism's ATCase, is ground for speculation and thus further study, since if it would be considered as the main component of the ATCase holoenzyme, this would make the enzyme's total mass to be 570 kDa, far too large when compared to the activity gel results. The results of the HIC chromatography column also suggest, that the *M.(B.) catarrhalis* 25240 ATCase is predominantly hydrophobic in nature, since as explained previously, it requires relatively low ammonium sulfate concentration, in the 0.1-0.2 M ammonium sulfate range, to elute from the column in its active form.

To summarize:

- a. This study has confirmed, based on the activity gel non-SDS PAGE, that *M.(B.) catarrhalis* ATCase holoenzyme has a molecular mass of about or above 480 kDa that it shares with the genus *Pseudomonas*; related species in the genus *Moraxellae* spp.

such as *M. bovis* and *M. osloensis*, respectively, also possess ATCases of the same size as *M.(B.) catarrhalis*. The *Moraxella* genus ATCase can thus be placed within the Class A of ATCases;

- b. This study has inferred preliminary from the 62% ammonium sulfate cut results and then, more concisely from the HIC chromatography, that the *M.(B.) catarrhalis* ATCase has to be predominantly hydrophobic in nature, eluting at 0.1M-0.2 M ammonium sulfate concentration, pH=8.0;
- c. This study has partially confirmed, based on the 4%-10% SDS-PAGE, that the *M.(B.) catarrhalis* ATCase holoenzyme is composed of two subunits, of 40.0 kDa and 45.0 kDa, respectively, although previous independent study has found the smaller subunit to be about 35 kDa in size. According to the Class A model, to which the *M.(B.) catarrhalis* ATCase has proven to belong, the smaller 40 kDa polypeptide subunit would seem to play a catalytic role in the enzyme function, while the larger one of 45 kDa serve as structural support of the holoenzyme architecture. This assumption seems to be confirmed by the HIC gel results, where the 40 kDa band persists with strong intensity at the 0.2- 0.1 M salt range while the 45 kDa disappears at the end of this range, being strongest only at 0.5 M salt concentration. Considering the relevant bands, 40 and 45 k Da polypeptides respectively, the total mass of the ATCase holoenzyme would amount to 510 kDa. The 55 kDa polypeptide, that co-runs with the previous ones, may be considered an accessory subunit component of the holoenzyme whose precise role has yet to be determined or a contaminant. Incidentally, it has been found in the same lab, that *Moraxella bovis* ATCase subunit composition also exhibit

a three-band pattern, of approximate size range from 40 kDa to 60 kDa that co-run on the SDS-PAGE gel.

- d. This study has shown that *M.(B.) catarrhalis* ATCase has to possess predominantly positive charges because it elutes at NaCl concentration of 0.196 M.
- e. Electroblothing has proven unsuccessful in numerous attempts to transfer the purified *M.(B.) catarrhalis* protein. Table #.1 on page 52, indicates that the purified protein concentration is 4.42 $\mu\text{g}/\mu\text{L}$ or 4.42 mg /mL, which is insufficient for proper detection on the PVDF membrane which requires at least 150 $\mu\text{g}/\text{cm}^2$ of protein material.

Hence, our study has been unable to obtain a N-amino acid sequence determination of the *M.(B.) catarrhalis* 25240 aspartate transcarbamoylase. One of the plausible reasons is that the ATCase enzyme has been derived from cellular extracts of the *M.(B.) catarrhalis* where the amounts of this particular enzyme that the cell naturally produces, are low. One of the methods to remedy the situation would be to clone the DNA segment containing the gene for ATCase into a vector capable of enhanced expression of the gene of interest, using a strong promoter, and obtain enough of the protein product for further manipulations and study.

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
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