

***Molecular Characterization of the
ATPase component of a putative ABC
type transporter in Moraxella catarrhalis***

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To my dad...

Abstract

Molecular Characterization of the ATPase component of a putative ABC type transporter in *Moraxella catarrhalis*

Moraxella catarrhalis is one of the major causes of respiratory tract and middle ear infections. Little is known about the pathogenesis of *M. catarrhalis* infection. A pathogenic bacterium requires adherence, invasion, establishment and dissemination with the host for a successful infection. The interaction between the host and the pathogen also includes uptake and secretion of substances that are facilitated by a family of proteins termed transporters. ATP-binding cassette (ABC) transporter is one of the active transport system common in prokaryotic and eukaryotic cells. In this study, we identified a gene encoding ATPase component of the ABC type transporter in *M. catarrhalis* and performed a nucleotide and deduced amino acid sequence analysis of this gene. We used Genomic Survey Sequence approach (GSS) to identify the gene from *M. catarrhalis* genomic library. The insert size of the positive clone is 934 bp and was sequenced by DNA walking. The ATPase domain of an ABC type transporter has conserved motifs: Walker A, Q loop, C loop (signature sequence), Walker B, D loop and Switch II. The ATPase domain is considered as the most conserved domain of the ABC type transporter due to presence of these motifs. Based on the homology and motif search, and amino acid sequence alignment, performed by using BLAST programs available on the internet, it was found that the deduced amino acid sequence encoded by this gene exhibited 50-80% identity with the sequences of the ATPase component of the ABC transporters of several pathogens.

Keywords: *Moraxella catarrhalis*, ABC type transporter, ATP-binding cassette transporter, ATPase domain, Walker A, Walker B.

ÖZET

***Moraxella catarrhalis*'te bulunan muhtemel bir ABC taşıyıcısının ATPase komponenti'nin Moleküler Karakterizasyonu**

Moraxella catarrhalis, solunum yolu enfeksiyonları ve orta kulak iltihaplarının en büyük etkenlerinden biridir. *M. Catarrhalis*'in hastalık yapıcı özelliği hakkındaki şu ana kadar edinilen bilgiler çok az.

Patojenik bir bakterinin konakladığı canlıda enfeksiyona yol açabilmesi için, konakladığı canlının hücrelerinin yüzeyine yapışması birinci koşuldur. Konak canlının hücrelerine yapışıp çoğalan patojen; ardından tüm vücuda yayılarak canlıda bir enfeksiyona sebep olabilir. Patojen ve konak canlı arasındaki etkileşimi sağlayan olaylardan bir tanesi de , iki canlı arasında, bazı protein taşıyıcıları (transporter) aracılığıyla, sağlanan alış verişdir. Bunlardan ATP-binding cassette (ABC) transporter sistemleri hem ökaryotlarda hem de prokaryotlarda aktif transportta yer alır. Bu tez çalışmasında, *M. Catarrhalis*'de bulunan muhtemel bir ABC transporter'ın ATP komponentinin moleküler karakterizasyonu yapılmıştır. Bu proteinin DNA ve amino asit dizileri çıkarılmıştır. Bu genin karakterizasyonunda yapılan deneylerde esas alınan yaklaşım Genomic Survey Sequence (GSS) approach'tur. Elimizdeki pozitif klonda bulunan insert, DNA walking methodu kullanılarak sekanslanmıştır. Buna göre ATPase komponentinin yapısında bulunan korunmuş motifler şunlardır: Walker A, Q loop, C loop (signature sequence), Walker B, D loop and Switch II. ATPase komponentinin ABC taşıyıcılarında en korunmuş yapı olarak kabul edilmesinin sebebi yapısında bu motifleri barındırıyor olmasıdır. Amino asit dizisi, çokça kullanılan, güvenilir biyoinformatik programlarından biri olan BLAST veri tabanında taranmıştır. Buna göre klonumuza ait amino asit dizisi bu veri tabanında bulunan diğer patojen bakterilerdeki ATPase komponentleriyle % 50-70 arası homoloji göstermiştir. Böylece, klonumuza ait amino asit dizisinin ABC taşıyıcılarıyla olan yakınlığı istatistiksel verilerle ortaya konmuştur.

Anahtar Kelimeler: *Moraxella catarrhalis*, ABC protein taşıyıcısı, ATPase komponent, Walker A, Walker B.

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Table of Contents

Dedication.....	iii
Abstract.....	iv
Özet.....	v
Acknowledgements.....	vi
Table of contents.....	1
List of tables.....	3
List of figures.....	4
Introduction.....	9
ABC Type Transporters.....	9
Structure.....	11
Mechanism.....	13
<i>Moraxella catarrhalis</i>	13
MATERIALS AND METHODS.....	17
2.1 MATERIALS.....	17
2.1.1 Chemicals.....	17
2.1.2 Bacterial Strains	17
2.1.3 Enzymes.....	17
2.1.4 Cloning vectors	17
2.1.5 Commercially Available Kits	19
2.1.6 Apparatus and Equipment.....	19
2.1.7 DNA Size Marker	20
2.2 SOLUTIONS AND MEDIA.....	20
2.2.1 Agarose Gel Electrophoresis Solutions	20
2.2.2 Microbiological Media and Antibiotics	20
2.2.3 Solutions for competent cell preparation:	21
2.3 METHODS	21
2.3.1 Growth and Maintenance of Bacteria	21
2.3.1.1 <i>M. catarrhalis</i>	21
2.3.1.2 <i>E. coli</i>	22
2.3.2 DNA Experiments.....	22
2.3.2.1 Competent Cell preparation and <i>E. coli</i> transformation	22
2.3.2.1.1 Simple and Efficient Method (SEM) for Preparation of Competent Cells	22

2.3.2.1.2	Transformation of <i>E.coli</i>	22
2.3.2.2	Plasmid DNA Isolation	23
2.3.2.3	DNA Ligation Reactions.....	23
2.3.2.4	Restriction Enzyme Digestion	23
2.3.2.5	Primers	23
2.3.2.6	Polymerase Chain Reaction (PCR).....	25
2.3.2.6.1	PCR assay	25
2.3.2.7	Genomic DNA Isolation from <i>M. catarrhalis</i>	26
2.3.2.8	Phenol-Chloroform Extraction.....	27
2.3.2.9	Agarose Gel Electrophoresis.....	27
2.3.2.10	Isolation of PCR Product from Agarose Gels.....	27
2.3.2.11	DNA Sequence Analysis.....	28
2.3.2.12	Bioinformatic tools used for nucleotide and amino acid sequence analysis	28
RESULTS		30
3.1	Isolation of the clones	30
3.2	Nucleotide sequence	30
3.3	Amino acid sequence	31
3.3.1.....		32
3.3.1.1.....		33
3.3.2	Motif search results.....	35
3.4	PCR results.....	36
DISCUSSION		38
Future perspectives		40
References.....		41

List of Tables

Table 1.....	20
Table 2.....	20
Table 3.....	21
Table 4.....	22
Table 5.....	24
Table 6.....	27
Table 7.....	27

List of Figures

Figure 1.....	8
Figure 2.....	14
Figure 3.....	14
Figure 4.....	15
Figure 5.....	28
Figure 6.....	28
Figure 7.....	29
Figure 8.....	29
Figure 9.....	30
Figure 10.....	30
Figure 11.....	31
Figure 12.....	31
Figure 13.....	31
Figure 14.....	32

Introduction

ABC Type Transporters:

Bacterial membrane transport occurs via pores, phosphoenolpyruvate (**PEP**)-dependent phosphotransferase (PTS) and peptide transporter systems such as ATP-binding cassette (ABC) type transporters. An ATP-binding cassette (ABC) type transporter is a protein having a characteristic ATPase domain, which is responsible for specific, unidirectional matter transportation across a lipid bilayer by ATP hydrolysis. These ABC type transporters are among the most conserved and wide spread protein superfamily found in from microbe to man (Schmitt L 2000; Higgins CF 2001; Verdon G 1998;). For instance, nearly 5% of the *Escherichia coli* genome encodes components of ABC transporters (Linton KJ 1998; Higgins CF 2001; Verdon G 1998).

The ABC type transporters are also called as traffic ATPases (Higgins CF 1992). There are three reasons for this designation. First of all, the ATP-binding sites in all ABCs are very similar in amino acid sequence. This implies that the members of the family hydrolyze ATP during transport processes. Secondly, the family members can translocate a variety of substrates differs both in size and shape. Finally, the term traffic is used to state that the transport takes place in either direction (Doige CA 1993).

Bacterial ABC transporters generally consists of up to four separate protein subunits, whereas their eukaryotic counterparts are generally composed of a single polypeptide (Locher KP 2002). Mainly the ABC type transporters are made up of four domains. Two of them are called as transmembrane domains (TMDs). They form a pathway across the membrane through which solutes move. These domains consist of multiple membrane spanning segments (putative α -helices) contain the substrate binding sites. On the other hand, the other two domains are highly conserved nucleotide –binding domains (NBDs), i.e. the ATPase domain. The NBDs are located at the cytoplasmic face of the membrane. They couple ATP hydrolysis and substrate translocation. (Schneider E 1998; Detmers F 2001; Locher KP 2002; Higgins CF 2001).

In general we can divide ABCs into three groups: Importers are mainly the prokaryotic substrate binding protein-dependent (BPD) transporters and they provide essential nutrients to bacteria even if these substances are present in submicromolar concentrations in the

environment. Exporters are found in both prokaryotes and eukaryotes. They are involved in the extrusion of waste products, the secretion of extracellular toxins and the targeting of membrane components. The third class of ABC systems are not involved in transport. However, they are involved in cellular processes such as DNA repair, translation or regulation of gene expression. (Schneider E 1998; Schmitt L 2002; Higgins CF 2001; Dassa E 2001; Locher KP 2002).

The ABC type transporters have a wide variety of physiological functions, including import of essential nutrients in prokaryotes, export of waste products out of the cell in both prokaryotic and eukaryotic cells. Additionally, they are involved in a large variety of processes, including signal transduction, protein secretion, drug and antibiotic resistance, antigen presentation, bacterial pathogenesis and sporulation.

The bacterial permeases are involved in solute uptake. They have been extensively analyzed and some shown to have homologous protein components with ATP-binding consensus sites (Fath MJ 1993). These permeases are members of an ABC superfamily, which includes the medically important eukaryotic multidrug resistance (MDR) protein and cystic fibrosis transmembrane regulator (CFTR) (Doige CA 1993). Members of this superfamily are very much alike in both sequence and structure; they have an ATP-binding motif (Doige CA 1993; Locher KP 2002). Moreover, in bacteria, multi-component permease consisting of one periplasmic substrate-binding protein, two hydrophobic cytoplasmic membrane protein with the ATP-binding motifs, forms a complex substrate binding protein-dependent transport system. This complex enables the bacteria to transport nutrients much more effectively (Schneider E 1998; Dassa E 2001; Mourez M 1997).

On the other hand, all of the eukaryotic ABC type transporters have their ATP-binding domain on a single polypeptide with the TMDs. Several of these ABCs have significant medical importance and since their discovery they have been studied very much in details. Among these, the ABC transporter MDR1 is responsible for the resistance of tumor cells to chemotherapy, and mutations in the ABC transporter, CFTR is the main cause of the most frequently occurring deadly inherited disease, cystic fibrosis (Gottesman MM 2001; Schmitt L 2000; Schmitt L 2002; Dassa E 2001; Verdon G 1998; Chang G 2001; Higgins CF 2001; Young J 1999). The Transporter associated with antigen processing protein (TAP) is another clinically important relevant ABC transporter, which is responsible for translocation of antigenic polypeptides from cytoplasm into endoplasmic reticulum (ER). In the ER they are being loaded onto major histocompatibility complex (MHC) class I molecules (Schmitt L 2000; Detmers F 2001; Dassa E 2001; Locher KP 2002).

As mentioned above, the highly conserved motifs of ATPase domain are the common feature of all members in the ABC superfamily. Many of these motifs, such as P-loop or Walker A motif; the Walker B motif, a glutamine residue in the Q loop, and a histidine residue in the Switch region, are directly involved in the binding and hydrolysis of ATP. In addition, ATPase domain has a D loop and a short polypeptide stretch (...LSGG...), which is so specific to this protein class that is generally called as “ABC signature sequence” (Gottesman MM 2001; Saurin W 1999; Doige CA, 1993; Locher KP 2002; Higgins CF 1992).

Structure:

The first high-resolution structure of an ATPase domain, HisP, the ATP-binding domain of histidine permease isolated from *Salmonella typhimurium* (Figure 1a), was reported in 1998 (Higgins CF 1982; Schmitt L 2002). The structure of HisP enables the scientists to understand the properties of ABC transporters and of defective CFTR proteins (Verdon G 1998). The HisP showed a novel, two-domain architecture. Domain I, the catalytic domain, has an α/β structure and contains the nucleotide-binding site, which is formed by a Walker A motif that precedes helix 1 plus the first three residues of this helix. In addition to this frequently encountered sequence (which is observed in many ATPases and GTPases), arm I contains the Walker B motif, is located in strand 9, and a conserved histidine, which acts as a γ -phosphate sensor, in the so-called ‘switch II’ region between strand 10 and helix 7. A sequence alignment of the ABC domains for which structures have been solved is shown in Figure 1b (Schmitt L 2002).

A conserved glutamine-rich sequence (residues 154-162 in His P; bacterial consensus sequence LSGGQQQRV), called the ‘linker peptide, ‘signature motif’ or ‘ motif C, is located in arm II in the crystal structure, with part of it forming the first half of α -5 and with residues 154-158 only being partially exposed. Several mutations of this motif inactivate the permease activity indicating that the motif performs an essential function. It is possible that the motif is necessary for the integrity of the folded HisP molecule (Verdon G 1998).

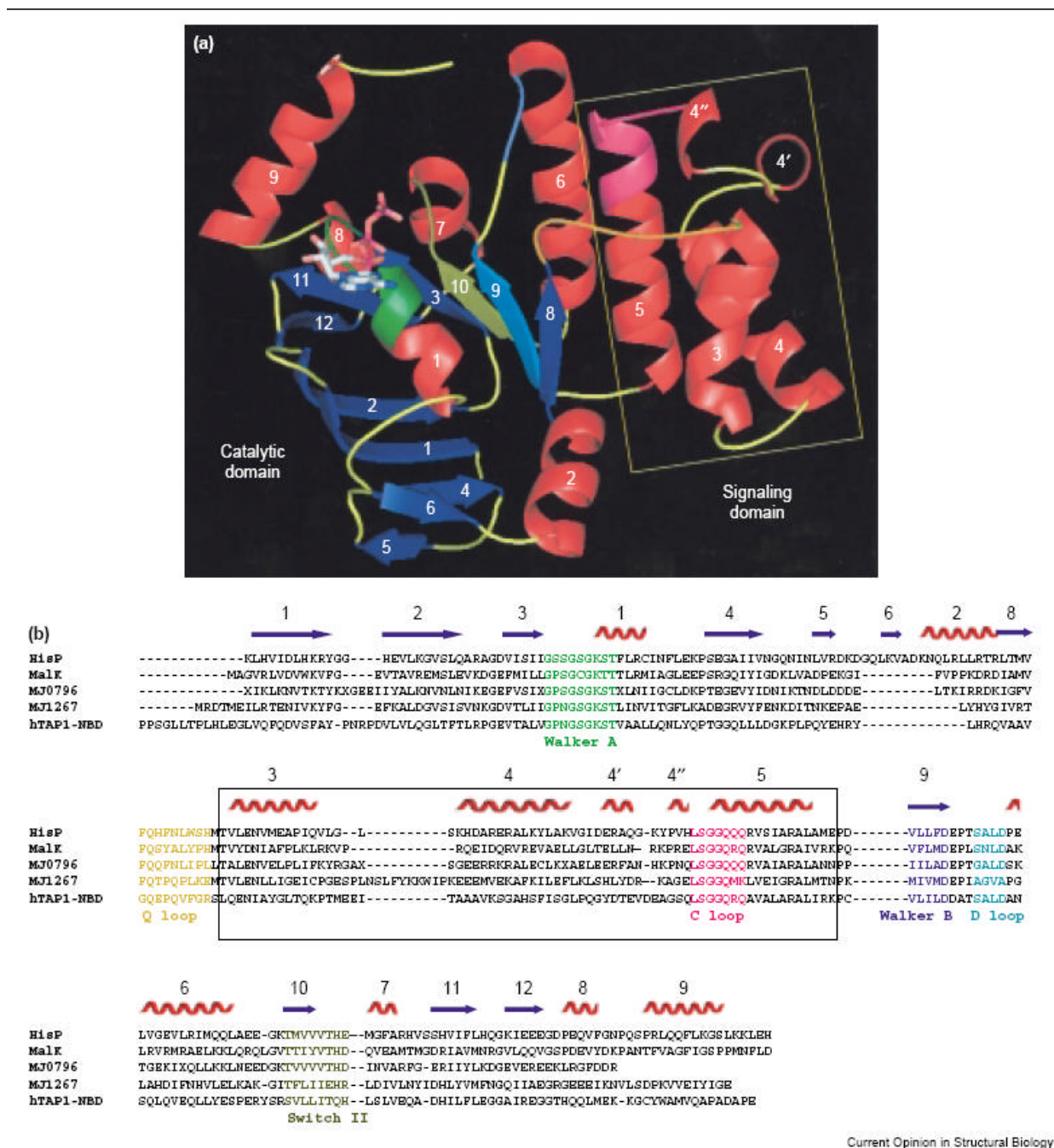


Fig.1) ATPase domain: structure-sequence conservation. **(a)** Structure of HisP. Helices are shown in red, strands are shown in blue and ATP is shown in ball-and-stick representation. **(b)** Sequence alignments of all reported X-ray structures of ATP-binding domains. Conserved motifs are colored and labeled. Secondary structure elements indicated above the alignment are from HisP (Schmitt L 2002).

Mechanism:

It is generally assumed that all ATPase domains bind and hydrolyze ATP in a similar fashion and use a common mechanism to power the translocation of substrate through the membrane-spanning partner domains. According to the current view of the mechanism begins with binding of the substrate to the transporter. Importers in gram-negative bacteria generally require a periplasmic binding protein that delivers the substrate to the periplasmic side of the transporter, whereas exporters recruit their substrates directly from the cytoplasm or, in the case of hydrophobic substances, from the inner leaflet of the plasma membrane. The binding event is signaled to the nucleotide hydrolysis sites, where it is thought to increase the affinity for ATP, a prerequisite for a productive transport cycle. The two ATPase domains then carry out the “power stroke”, a highly cooperative ATP-binding and hydrolysis reaction that is concurrent with a substantial conformational change. This change is coupled to mechanistically critical rearrangements in the membrane-spanning domains associated with unidirectional substrate translocation, which is believed to occur through a tailored pathway at the domain interface. After the substrate has crossed the membrane, the transporter returns to the resting state through the dissociation of ADP and inorganic phosphate (Locher KP 2002).

Moraxella catarrhalis:

Moraxella (Branhamella) catarrhalis, formerly called *Neisseria catarrhalis* or *Micrococcus catarrhalis* is a gram-negative aerobic diplococcus that is frequently found as a normal inhabitant of human respiratory tract. This bacterium has emerged as a pathogen over the last 20 years, (Johnson 1981). However, now it is considered as an important cause of respiratory tract infection (RTI) and otitis media together with *Haemophilus influenzae* and *Streptococcus pneumoniae*.

M. catarrhalis had been known as a harmless commensal for a long time in this century. The reason for how or why suddenly emerged, as a pathogen is not yet known. However, the followings might be considered as major reasons:

1. The bacterium may have changed such that it is now more virulent than in past decades.
2. Since *M. catarrhalis* has long been regarded as a nonpathogenic commensal, infections by the organism may have been occurring but have been overlooked for years.

Since the infections *M. catarrhalis* causes results in a high level of morbidity and mortality and a substantial financial burden, It is important to explore this organism. For instance, it is responsible for about 15-20 % of all otitis media cases in children, in USA (Enright 1997). This results in a substantial financial burden (\$2 billion/year in US) on the health care system (Murphy 1996).

Although, it is accepted that *M. catarrhalis* is an established pathogen all over the world, its virulence factors remain to be discovered. Moreover, the pathogenesis of this bacterium is not yet clear, either. In addition, substantial increase in β -lactamase producing *M. catarrhalis* makes it difficult to treat with mostly used antibiotics (Martinez, 1998). Therefore, it is urgent to find new preventive and therapeutic strategies to fight with the infections caused by this bacterium. In addition to all these, the complete genome sequence of *M. catarrhalis* is not known.

The outer membrane proteins (OMPs) of *M. catarrhalis* are similar to other gram-negative bacteria. Similar to them 10 to 20 OMPs are present with 6 to 8 of these proteins predominating (Murphy 1990). The outer membrane of *M. catarrhalis* contains 8 major OMPs that are named beginning with OMP A to OMP H. The molecular weight of these proteins ranges from 98 to 21 kDa. It is interesting that there is a significant homogeneity among OMPs of 50 strains. This means that an immune response against one strain will also recognize a subsequent strain (Bartos 1988).

The list of the major OMPs of *M. catarrhalis* is given below:

CopB:

One of the first OMP studied was CopB. It shows moderate degree of antigenic conservation when compared to other strains tested (Karalus 2000). Evidence for saying that an antigen is conserved:

- Antibody reactivity
- Restriction Fragment Length Polymorphism (RFLP)
- Comparison of sequence of the gene

The protein is immunogenic as the antibody against it could be detected in convalescent sera of patients (Sethi 1995). Moreover, passive immunization of mice with the antibody against CopB resulted in enhanced clearance of *M. catarrhalis* from the lungs of these animals (Karalus 2000). Mutation and expression analysis show that CopB is involved in iron acquisition. Iron is an important element for many bacteria to live on their mammalian hosts.

Therefore bacteria developed strategies to transfer low concentration of iron from their environment.

OMP CD:

Sequence analysis and RFLP studies on OMP CD show high degree of conservation among different strains of *M. catarrhalis*. It is a heat-modifiable protein that was initially reported as OMP C and OMP D. Then it was realized that these two bands were the two forms of a single antigen. BLAST search shows that the protein has homology with *Pseudomonas aeruginosa* porin protein (Murphy 1993). The antibody against OMP CD elicit complement-mediated bactericidal activity against various strains of *M. catarrhalis* which indicate it may stimulate a protective immune response if used as a vaccine.

UspA1 and UspA2:

They were first named as High Molecular Weight OMP (HMW-OMP) since the protein forms oligomeric complexes. Later on it was realized that the complex was composed of two proteins and named as ubiquitous surface protein A1 and A2 (UspA1 and UspA2). They were characterized as a single protein not only because of a single band in Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) but also because they share a common epitope. In fact they share a common 140-aminoacid region that is 93% identical (Cope 1999). In addition, passive immunization of mice with the antibody against CopB resulted in enhanced clearance from the lungs of these animals (Karalus 2000).

Fimbriae:

Fimbriae have been described on the surface of *M. catarrhalis* by electron microscopy (Ahmed 1990). In *M. catarrhalis*, fimbriae are responsible for adherence and hemagglutination, therefore thought to be an important virulence factor (Ahmed 1992a). It was also found that fimbriae are present on all fresh isolates of *M. catarrhalis*, and it decreases in number upon repeated in vitro passages (Ahmed 1992). Since fimbriae has not been isolated, little information available regarding the characterization of these structures.

Lipooligosaccharide(LOS):

Another prominent bacterial surface component of *M. catarrhalis* is the LOS. *M. catarrhalis* LOS shares homology with LOS of other gram-negative bacteria. In addition, these shared LOS epitopes have been implicated as a virulence factor for *M. catarrhalis* i.e. resistance

to bactericidal activity mediated by normal human serum (Zaleski 2000). There are three major LOS serotypes in *M. catarrhalis*: A, B and C. these structures encompass 95% all strains studied to date (Rahman 1996). Recently, it has been suggested that LOS may act as an adhesin of *M. catarrhalis* (Hu W-G 2001).

As in all prokaryotes, ABC type transporters involve in many cellular transport processes in *M. catarrhalis*. In some cases they might be responsible for bacterial resistance to antibiotics. By using the genomic survey sequence approach (GSS) (Rosinha GM, 2002) in this project the ATPase domain of an ABC type transporter in *M. catarrhalis* was identified.

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

All chemicals were of analytical grade and supplied by Sigma-Aldrich Chemie GmbH (Germany) except for:

- Ampicillin was purchased from Roche Diagnostics GmbH (Germany).
- IPTG and X-Gal were purchased from MBI Fermentas Inc. (USA).

2.1.2 Bacterial Strains

For all purposes *M. catarrhalis* B-88-152 isolated from sputum of patients with RTI and for cloning purposes *E. coli* DH5 α were used. The *E. coli* strain was used in library construction and propagation of plasmids. The manufacturer is Bio-Rad (Hercules, USA).

2.1.3 Enzymes

The restriction enzyme *Hind*III, the DNA modifying enzyme, T4 ligase and *Taq* polymerase were purchased from MBI Fermentas Inc. (USA).

2.1.4 Cloning vectors

The pQE-31 (Qiagen Inc. USA) and pBluescript SK (Stratagene Inc. USA) vectors were used in colony hybridization experiments, as the pGEM T-Easy vector, Promega (USA) was used in cloning of PCR products.

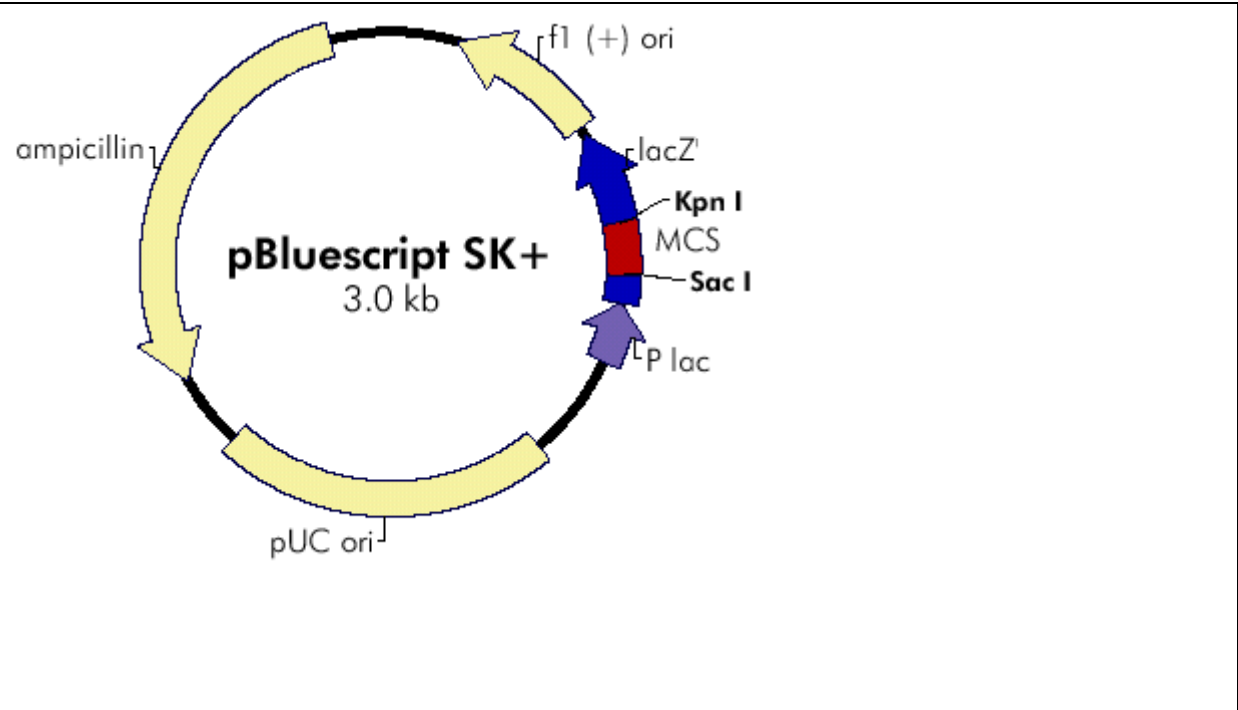


Figure 2) The map of pBluescript cloning vector. It contains *lacZ* gene and suitable for α -complementation.

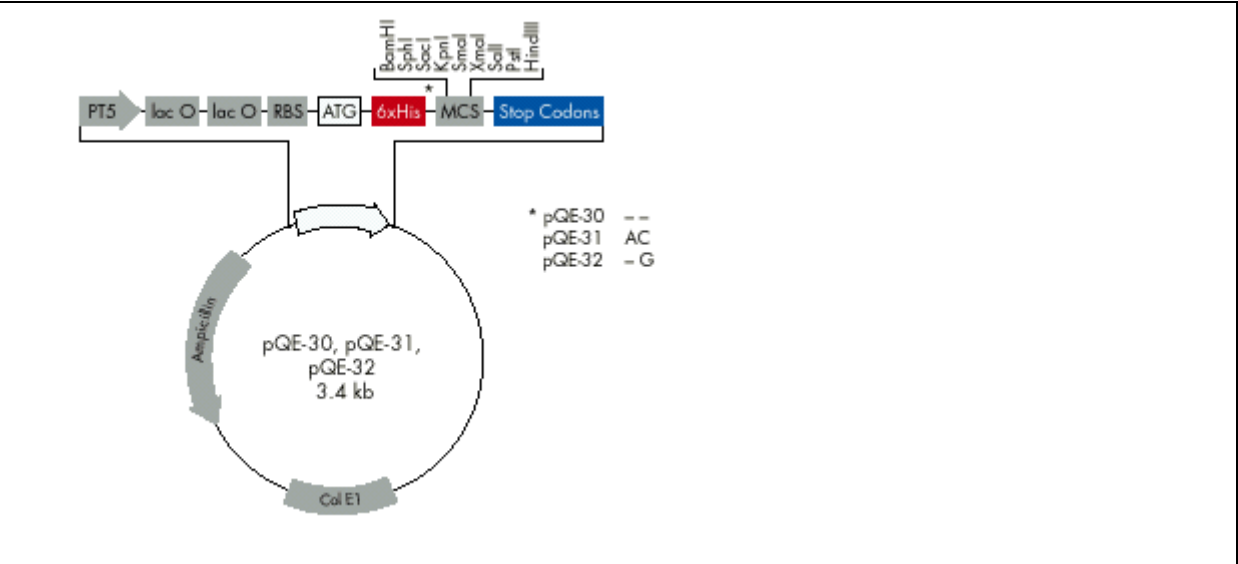
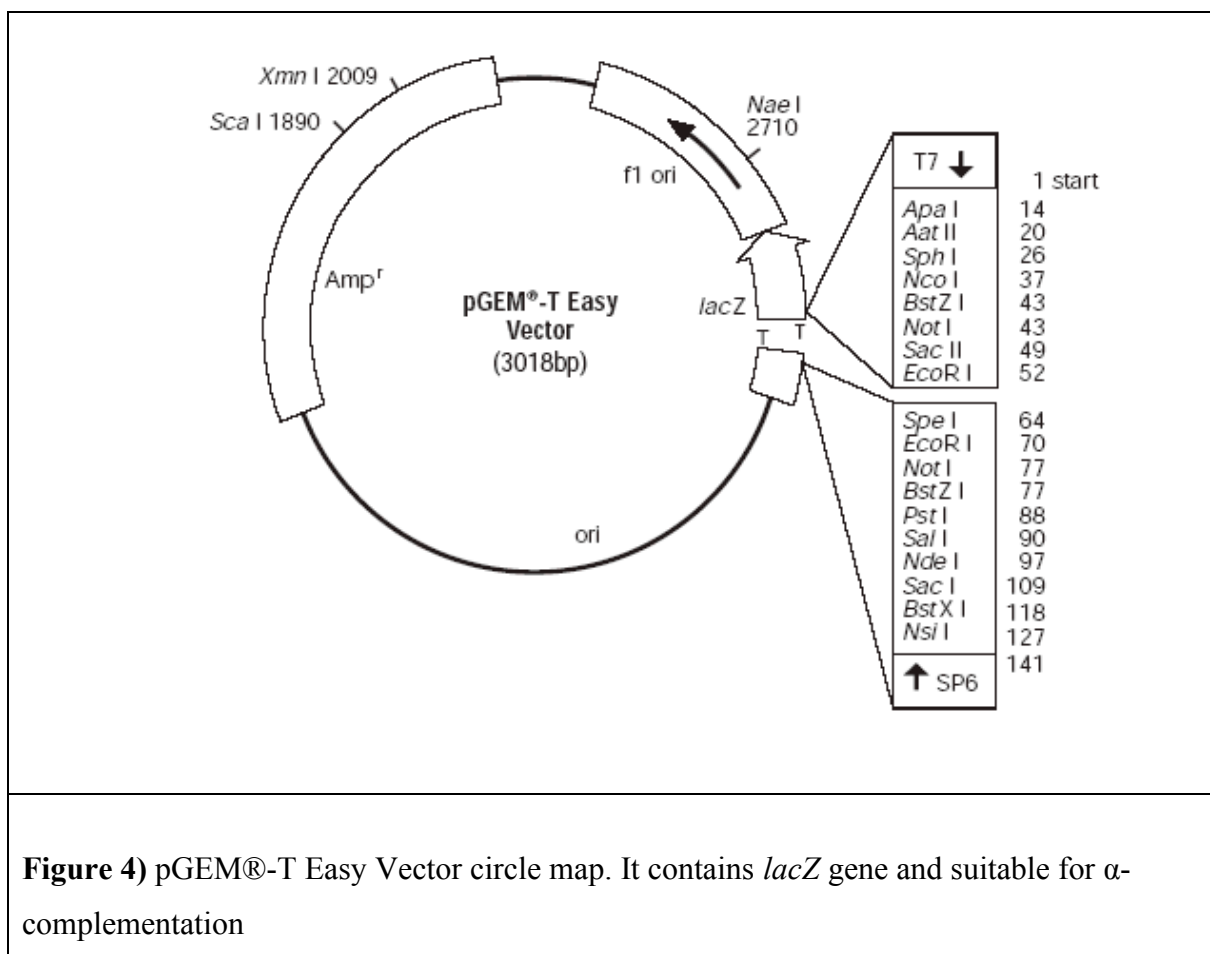


Figure 3) The map of pQE expression vector. It contains *lac* operon and is suitable for expression studies.



2.1.5 Commercially Available Kits

- Qiaex II Gel Extraction Kit (by Qiagen Inc., USA) was used to purify PCR product from agarose gels.
- MN Nucleospin Plasmid (Macherey-Nagel, GmbH & Co, Germany) was used in isolation of plasmids from *E.coli*.
- DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences UK Limited, England) was used for sequencing reactions.

2.1.6 Apparatus and Equipment

Agarose gel electrophoresis were done by using horizontal mini gel apparatus Mupid-2 purchased from Advance Co., Ltd., Japan. Thermal cycler for PCR was a product of Perkin Elmer (USA).

2.1.7 DNA Size Marker

1kb DNA ladder from MBI FERMENTAS Inc. (USA) was used as DNA size marker.

2.2 SOLUTIONS AND MEDIA

2.2.1 Agarose Gel Electrophoresis Solutions

50X Tris-Acetic acid-EDTA (TAE)

Per Liter: 242 g Tris Base

57.1 ml Glacial Acetic acid

100 ml of 0.5 M EDTA

Ethidium bromide

10 mg/ml in water (stock solution)

30 ng/ml (working solution)

2.2.2 Microbiological Media and Antibiotics

Luria-Bertani Medium (LB)

Per Liter: 10 g Bacto tryptone,

5 g Yeast extract

5 g NaCl

15 g Agar (for solid media)

Blood Agar

%7 Rabbit blood in BHI agar

Brain Heart Infusion (BHI) Agar

Per Liter: 52 g of BHI agar powder

BHI Broth

Per Liter: 37 g of BHI broth powder

Mueller Hinton (MH) Broth

Per Liter: 21 g of MHB powder

Ampicillin

100 mg/ml (stock solution)

100 µg/ml (working solution) in dH₂O

2.2.3 Solutions for competent cell preparation:

SOB medium (100ml) (Sterilized by autoclaving)	2% Bacto-tryptone 0.5 % yeast extract 10 mM NaCl 2.5 mM KCl 10 mM MgCl ₂ 10 mM MgSO ₄
Transformation Buffer (TB) (Sterilized by filtration through 0.45µm filter, stored at 4 °C)	10mM Pipes 55mM MnCl ₂ 15mM CaCl ₂ 250mM KCl pH 6.7
SOC	SOB with 20mM glucose

2.3 METHODS

2.3.1 Growth and Maintenance of Bacteria

2.3.1.1 *M. catarrhalis*

The stocks of the *M. catarrhalis* strains were done in MH broth containing 5% rabbit blood and stored at -20°C until use. Prior to stocking, the bacteria were cultured on 7% rabbit blood agar plates. Then, the colonies were taken with a sterile cotton swab and transferred into the 1 ml of media contained in a cryotube. During experiments, after cultured on BHI agar, the bacteria were incubated within a box containing 5% CO₂ at 37 °C. The CO₂ generated by the CO₂ Gen Compact (Oxoid Limited, UK). All media were purchased from Merck KGa (Germany).

2.3.1.2 *E. coli*

E. coli strains were stored in 50% sterile glycerol at -70 °C till it was used. The stocking was done as following: Overnight grown cultures were mixed with sterile glycerol in a ratio of 1:1, mixed for homogenization and kept at -70 °C. During the experiments the *E. coli* were cultured either on LB agar or in LB broth. The broth cultures were incubated in a rotary shaking incubator at 37 °C with a speed of ~200 rpm. Similarly, LB agar plates were incubated overnight at 37 °C incubator.

2.3.2 DNA Experiments

2.3.2.1 Competent Cell preparation and *E. coli* transformation

2.3.2.1.1 Simple and Efficient Method (SEM) for Preparation of Competent Cells

A single colony of appropriate *E. coli* DH5 α strain was inoculated into 15 ml of LB broth (containing the appropriate antibiotics which is ampicillin in this study) and grown overnight at 37 °C. Then, the starter culture was diluted to OD₆₀₀ of 0.2-0.3 into 250 ml of SOB medium and grown until the OD₆₀₀ of the culture reaches to 0.6 at 18 °C with a speed of 200-250 rpm shaking. It is very important that the aeration of the culture during growth is well. For this grow the culture into a wide flask closed with a sterile cotton cap. After that, the culture was chilled on ice for 10 minutes, and centrifuged at 2500 x g for 10 minutes at 4 °C. The pellet was resuspended in 80 ml of ice-cold TB, and an other 10 minute incubation on ice was done. The mixture was pelleted as mentioned above, resuspended gently in another 20 ml of TB. After that, DMSO was added to a final concentration of 7%, mixed gently and incubated on ice for 10 minutes for the last time. Subsequently, the aliquots of this mixture were done and these aliquots were chilled in liquid nitrogen, and then stored at -70 °C up to 3 months without loss of transformation efficiency (Inoue et al. 1990).

2.3.2.1.2 Transformation of *E.coli*

An aliquot of frozen competent cells was thawed on ice. 200 μ l of the cells was mixed with plasmid DNA with a concentration of less than 100 ng/ μ l in a 1.5 ml eppendorf tube and incubated on ice for 30 minutes. The cells were then exposed to 42 °C for a heat shock for 90 seconds and chilled on ice for 2 minutes after the heat shock. 800 μ l of SOC medium was

added and the cells were grown at 37 °C for 45 minutes with vigorous shaking at 250 rpm. The culture was then centrifuged and the pellet was resuspended in 100 µl of SOC, and spread on LB agar plates containing the appropriate antibiotic, which is ampicillin in this study.

2.3.2.2 Plasmid DNA Isolation

The plasmid, was isolated with MN Nucleospin Plasmid kit according to instructions of the manufacturer.

2.3.2.3 DNA Ligation Reactions

Ligation of the DNA fragments into plasmid vectors were done with 0.3-1.0 µg of linearized plasmid vector and 3-5 times molar excess of insert DNA in the presence of 1-4 Weiss units of T4 ligase and 1x concentration of the standard ligation buffer supplied by the T4 DNA ligase. And the mixture was incubated overnight at 16 °C. The reaction volume was 15µl.

2.3.2.4 Restriction Enzyme Digestion

In restriction enzyme digestion 5 µl of plasmid (1-10 µg/µl), 2 µl restriction enzyme buffer, and 1 µl restriction enzyme, *HindIII*, was mixed in small tubes and the reaction volume was adjusted to 20 µl with sterile ddH₂O.

2.3.2.5 Primers

The primers for both sequencing and PCR reactions were designed by using the Primer3 program. The primers designed for PCR reactions were used in the sequencing reactions as well. The criteria for proper primer design are given below.

- T_m (the melting temperature of the primer) in the range of 52 °C to 65 °C
- Lack of dimerization capability
- Lack significant hairpin formation (>3 bp)
- Absence of secondary priming sites
- Lower GC content as much as possible to avoid mispriming.

Generally the lengths of the primers used in this study were 19bp or 20 bp. Moreover, their GC content is ranging from 40 % to 55 %. All of the primers designed worked properly (Table 1 and Table 2). All primers used in this study were custom synthesized and purchased from Iontek (Bursa, Turkey).

In addition, the primers designed for the first PCR reaction were constructed from the most conserved amino acid sequences of Walker A motif of several organisms, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Agrobacterium tumeficiens*, and *Brucella melitensis*, that insert B5 has significant homology. In addition to the most conserved amino acid sequence of the Walker A, gram negative codon usage was considered as ABC1 was designed; as the ABC2 primer was designed according to universal codon usage, and finally ABC3 was constructed by using universal codon usage, but in this case the primer has degeneracy (Table 2).

KA2: 5'-TGCAGGTCTTGAAAATGCAG-3'
B5 2: 3'-TGGATGAGCTGCTTGATTTG-5'
T3: 5'-AATTAACCCTCACTAAAGGGA-3'
T7: 5' TAATACGACTCACTATAGGG 3'
Table 1) Primers used in sequencing reactions

B5 2: 3'-TGGATGAGCTGCTTGATTTG-5'
ABC1: 5'-AACGGCAGCGGCAAAAGCAC-3'
ABC2: 5'-TGGCCTAGTGGTAGTGGTA-3'
ABC3: 5'-GGYCCYAGYGGYAGYGGYA-3'
Table 2) Primers used in PCR assays

2.3.2.6 Polymerase Chain Reaction (PCR)

PCR was done in order to find out the Walker A motif in the putative ATPase domain of ABC transporter in *M. catarrhalis*. The reaction conditions and procedure for PCR are given below. In general all of the reagents were delivered in a 1.5 ml eppendorf tube and a master mix was prepared on ice. In addition, before and after giving the *Taq* polymerase, the master mix was mixed gently by vortexing.

The master mix was distributed into PCR tubes with a volume of 48 μ l. Then, 2 μ l of *M. catarrhalis* genome template DNA was given into tubes. For negative control instead of genomic DNA, 2 μ l sterile ddH₂O was added. The PCR products were run on 0.8 % or 1.0 % agarose gel containing EtBr for visualization under UV lamp.

2.3.2.6.1 PCR assay

Three sets of PCR assays were done with three different forward primers (ABC1, ABC2 and ABC3), one for each set, and one reverse primer (B5-2), for all sets. The reaction volume in each PCR tube was 50 μ l and the reaction cycle was 35.

Reagents	Final concentration	Quantity for 50 μ l of reaction mixture
ddH ₂ O	-	33.1 μ l
10x PCR buffer	1x	5 μ l
25 mM MgCl ₂	1.75 mM	2.5 μ l
2 mM dNTP mix	0.2 mM of each	5 μ l
Primer I (B5-2)	20 pmol/ μ l	1 μ l
Primer II (ABC1/ABC2 /ABC3)	20 pmol/ μ l	1 μ l
<i>Taq</i> DNA polymerase	1.25 u/50 μ l	0.4 μ l
Template DNA	50 ng	2 μ l
Table 3) PCR assay mixture set up		

2.3.2.7 Genomic DNA Isolation from *M. catarrhalis*

Amplification steps	Temperature (°C)	Duration (min)
Initial denaturation	94	5
Denaturation	94	0:30
Primer annealing	56	0:20
Extending	72	1:10
Final extending	72	5
Table 4) The PCR conditions for amplification		

Large scale genomic DNA isolation was performed by the standard methods of Current Protocols for gram-negative bacteria. 100 ml BHI broth was inoculated with 1 or 2 colonies of *M. catarrhalis*. After overnight incubation the bacteria were harvested by centrifugation at 10000 rpm. The pellet was resuspended in 8.75 ml of 10x TE (100mM Tris-HCl, pH 7.5, 10 mM EDTA) by repeated pipetting and then transferred to a disposable 50 ml centrirtube. The cell suspension was frozen at -20 °C for 60 minutes. 1.0 ml of freshly prepared lysozyme (10 mg/ml in 0.25 Tris-HCl, pH 8.0) solution was added to the frozen cells and then thawed by mixing in a room-temperature water bath. Just after thawing, the cells were put on ice for 45 minutes. 0.25 ml of 20 % SDS, 50µl of 20mg/ml proteinase K and 10 µl of 10 mg/ml DNase-free RNase was added to the cell suspension. After mixing thoroughly the solution was incubated at 55 °C for 1 hour with occasional gentle mixing. Then 1.8 ml of 5 M NaCl was added and the tube was mixed thoroughly by inverting for 5 minutes. 1.5 ml of CTAB/NaCl solution was added and the tube was mixed thoroughly and then it was incubated at 65 C for 20 minutes with occasional shaking.

Approximately equal volumes of chloroform: isoamyl alcohol (49:1) was added, the tube was mixed thoroughly by inverting the tube for 5 minutes, and it was spin down 10 min at 3000 rpm. The chloroform phase was removed and the supernatant and the interphase were homogenized with a plastic pipette. The suspension was centrifuged 10 min at 3000 rpm again and the aqueous, viscous supernatant was transferred to a fresh centrifuge tube, leaving the interface behind. An equal volume of phenol:chloroform: isoamyl alcohol (25:24:1), was added and the solution was extracted thoroughly by inverting the tube and was centrifuged 10

minutes at 3000 rpm. The aqueous, viscous supernatant was transferred to a fresh centrirtube, leaving the interface behind. 0.6 volume of isopropanol was added to precipitate the nucleic acids. The DNA pellet was transferred to a fresh tube containing 70 % ethanol by hooking it onto the end of a glass Pasteur pipette. The DNA was washed with 70 % ethanol to remove the residual CTAB and recentrifuged to pellet it. The supernatant was removed carefully and the pellet was dissolved in TE.

2.3.2.8 Phenol-Chloroform Extraction

Phenol-chloroform extraction was done to purify DNA after each enzymatic manipulation. First, an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the DNA solution containing the impurities. The optimum starting volume was about 100 µl. If the solution was less than this volume it was diluted with ddH₂O accordingly. After mixing 45 seconds by vortexing, the tube was centrifuged for 5 minutes at 13000 rpm. The upper (water phase) was transferred to a new eppendorf tube. Then 1/10 volume of 3M NaOAc was added. After mixing by inverting the tube 2.5 volume of cold absolute ethanol was added. After mixing by inverting the tube was kept in -80C for 20 minutes. Then the tube was centrifuged for 10 minutes at 13000 rpm. After discarding the supernatant, the pellet was washed with 200 µl of 70% ethanol. After drying all the ethanol the pellet was dissolved in TE or water.

2.3.2.9 Agarose Gel Electrophoresis

DNA fragments of less than 1kb were generally separated on 1.0 % agarose gel, those greater than 1kb were separated on 0.8 % gels. Required amount of agarose gels were completely dissolved in 1x TAE buffer by heating in microwave, then ethidium bromide was added to a final concentration of 30 ng/ml. The DNA samples were mixed with one volume loading buffer and loaded onto gels. The gel was run at room temperature in 1x TAE at 50 V or 100 V.

2.3.2.10 Isolation of PCR Product from Agarose Gels

The kit given in materials part was used in isolation of PCR product. We followed the instructions of the manufacturer.

2.3.2.11 DNA Sequence Analysis

DNA sequence analysis of the positive clones was performed by the dideoxy chain termination method. Cycle sequencing reactions were set up in 0.2 ml eppendorf tubes. The sequencing procedure was performed by DYEnamic ET Terminator Cycle Sequencing Kit according to the instructions of the manufacturer. The cycle sequenase reactions were performed in Perkin Elmer GeneAmp PCR 9600 system thermal cycler with the following parameters: 25 cycles of denaturation (95 °C for 20 seconds), annealing (50 °C for 15 seconds) and extension (60 °C for 1 minutes). Electrophoresis was performed on the ABI PRISM™ 377 Genetic Analyzer (Perkin Elmer, USA) by loading the samples into the lanes of a vertical polyacrylamide slab gel. The separated DNA fragments were exposed to a laser which in turn would excite the flourescent dyes attached to the fragments. These data were then collected and analyzed by the help of ABI Sequencing Analysis Software (version 3.0.1).

In addition, for complete nucleotide sequencing new primers were designed and the procedure mentioned above was used in subsequent sequencing reactions.

Sample	Template (500ng) (µl)	Primer name	Primer (2pmol/µl) (µl)	Mix (µl)	ddH2O (µl)
B5	8	T3	2	8	2
B5	8	T7	2	8	2
B5	8	KA2	2	8	2
B5	8	B52	2	8	2
Control	1	Control	2	8	7
Table 5) Protocol for DYEnamic ET Terminator Cycle Sequencing Kit					

2.3.2.12 Bioinformatic tools used for nucleotide and amino acid sequence analysis

For nucleotide sequence homology search “BLAST align two sequences” tool was used. For conserved domain homology search, “the conserved domain homology search” engine of BLAST was used. In addition, translation of the inserts’ nucleotide sequences into 6 phase amino acid sequence were done by the “justbio translator” tool and the complementary sequence of the inserts’ reverse sequences were obtained by using “justbio complementor

program”. Also, “justbio aligner” tool was used for two amino acid sequences alignments. Finally, for multiple amino acid sequence alignments “clustalW” program was used.

RESULTS

3.1 Isolation of the clones

In previous studies performed in our lab, a genomic library of *M. catarrhalis* was prepared in pBluescript SK (pBSK) and pQE 31 vectors after digesting the genomic DNA with *Hind*III using standard molecular biology methods. Then transformation of the genomic library was done in *E. coli* DH5- α strain. The library constructed by using the pQE 31 vector was screened by monoclonal antibody (mAb) against 55kDa protein, which is an adhesin protein, isolated from *M. catarrhalis* in our lab. In addition, the library constructed by using the pBSK vector was screened by an oligonucleotide probe deduced from N-terminal amino acid sequence of 55kDa protein.

A total of 12 positive clones (B5, C1, 1B1, 1B2, 1B3, 3A, 3B, 5A, 6A, 8A, 8B1 and 8B2) out of 4000 colonies were identified based on X-gal detection method. The size of the inserts of the clones ranged from 750bp to 1500bp (Turan T 2002).

3.2 Nucleotide sequence

We attempted to obtain the nucleotide sequence of the inserts of all of the clones (Table 6). Inserts of clones, B5, C1 and 8B2 were sequenced completely. On the other hand, we obtained the partial sequences of the inserts of the rest of the clones. Almost 70 % to 80 % of these inserts were sequenced. Among these clones the insert of the B5 showed significant homology with the ATPase domain of the ABC type transporter.

Name of the clone	Sequenced length of the insert
pQE 31 clones	
1B1	1719bp
1B2	929bp
1B3	1135bp
3A	1200bp
3B	838bp
5A	745bp
6A	1063bp
8A	1283bp
8B1	902bp
8B2	734bp (complete sequence of the insert)
pBluescript SK+ clones	
B5	934bp (complete sequence of the insert)
C1	1827bp (complete sequence of the insert)
Table 6) The names of different clones and lengths of the completed sequences of the inserts.	

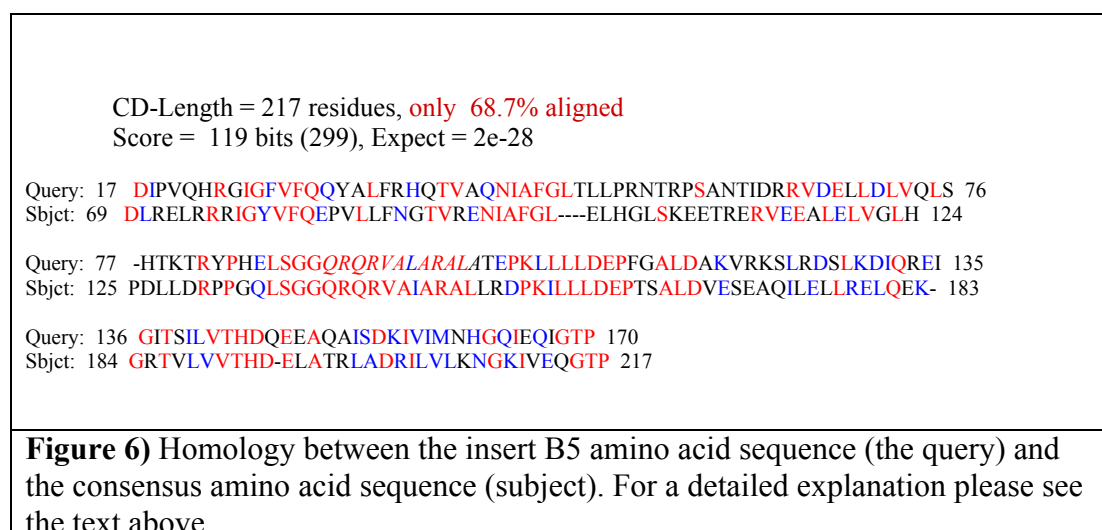
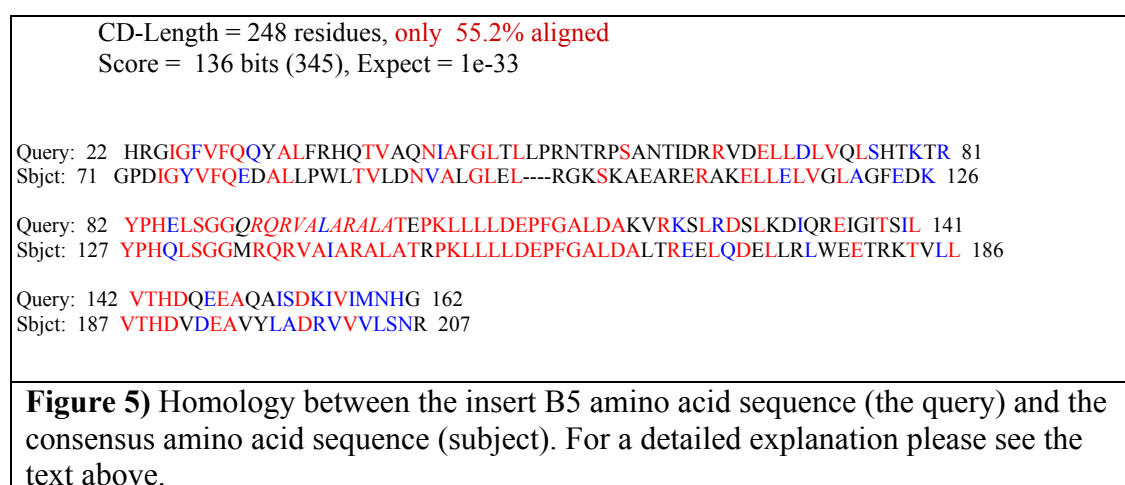
3.3 Amino acid sequence

LHYLP*LLWSLKD**AIKNSTDDILVK*DLHQNLHIFINLSKQLSSMSIKHEY*N
 PKHP*NFRGFCCLR*NQH*YFNR*ADNFIRSIRLWQDNFATHYCRS[KCRQRA
 GYILTVSNVTDIPVQHRGIGFVFQQYALFRHQTVAQNIAFGLTLLPRNTRPSA
 NTIDRRVDELLDLVQLSHTKTRYPHELSGGQRQRVALARALATEPKLLLLDE
 PFGALDAKVRKSLRDSLKDIQREIGITSILVTHDQEEAQASDKIVIMNHGQIEQ
 IGTPSKLFAQPSSDFVVDLGL]AMSHRSKINRHMVFNLH*SQX

Table 7) The converted amino acid sequence of the insert of clone B5. The motifs of ATPase domain are found in the region shown dark within the brackets []. “*” Indicates the stop codons.

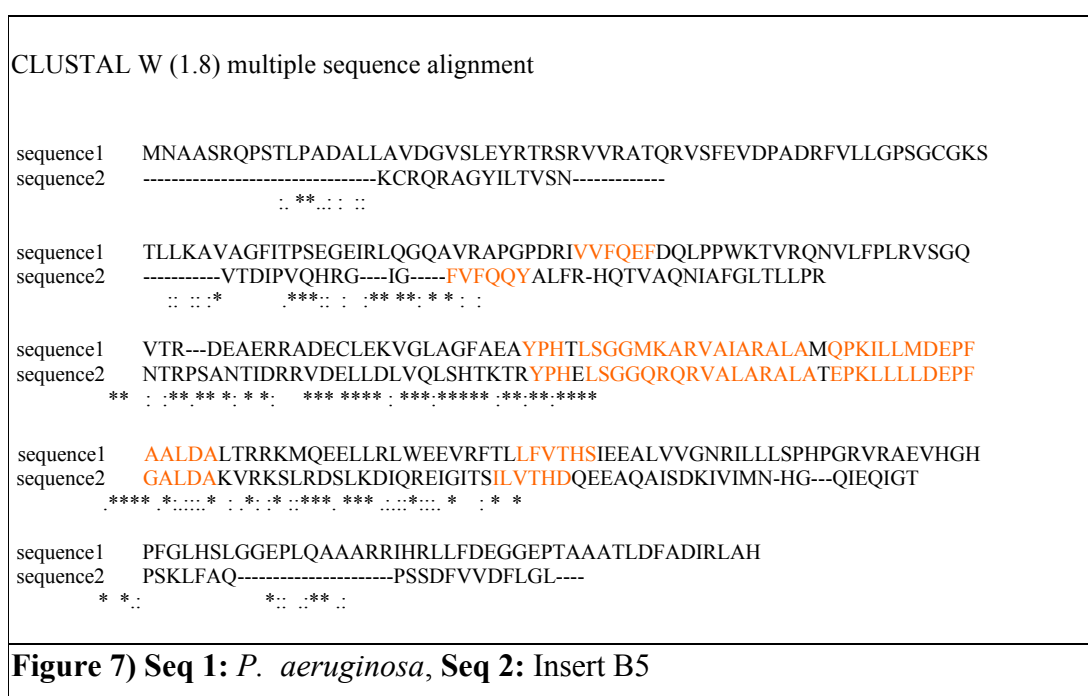
3.3.1

The conserved domain homology search was done in BLAST by using “conserved domain homology” search tool in BLAST. The second phase amino acid sequence of the insert B5 showed 50 % to 70 % homology with the ATP-binding domain, the ATPase of the ABC transporters of several bacterial species including, *N. meningitides*, *P. aeruginosa*, *H. influenzae*, *H. pylori* and *S. typhimurium* LT. Figures 8 and 9 below show where 55% homology and 68% homology were found respectively. In both of the tables the query sequence is the insert B5 and the subject sequence is the corresponding consensus sequence. In addition, the CD-length shows the conserved domain length, bit score that each alignment has is the measure between the hit, the sequences that produce “significant” alignments to the query sequence, and the query. The Expectation value (e-value) is "an assessment of the statistical significance of the score". Also, the e-value is showing how random a match is or how much chance is involved. As a result, a low e-value is an indication of a high homology.



3.3.1.1

Specific examples showing the most homologous amino acid sequences between the ATPase components of insert B5 amino acid sequence and several bacterial species: *P. aeruginosa*, *H. influenzae*, *S. aureus subsp. aureus N315*, *N. meningitidis serogroup B strain MC58*, *H. pylori*. In each figure, asterisks indicate identical residues, colons indicate conserved substitutions, and periods indicate semiconserved substitutions. In addition, the amino acids highlighted with orange indicates the regions those are most identical between the two sequences.



```

sequence1  MVRIQDLSLAFNGQTLFEHLNLTLLPNEWVSLLGSSGVGKSTLLRLLAGIETQGMVQGKI
sequence2  -----KCRQRAGYILTVSNTDIP-----VQHR-GIG--FVFQQYALFRHQTV AQN-I
          . . . : *.*.* : *.* :. *.*.*.*

sequence1  LFEPKVRIAWLPQKETLYPWLSIVDNVQLQAVLFGKRKSVKTTTEKAKMLLEKVGMAAHWHK
sequence2  AFG---LTLLP-R-----N-TRPSANTIDRR-----VDELLDLVQLSHTKTR
          *  :: **:      :. :.* :.*  ..**.* :. :

sequence1  PCSQLSGGQKQRVALARTLMQEVDLILLDEPFSAIDISRHLQDLAFELLEDK--SVLL
sequence2  YPHLESGGQRQVRALARALATEPKLLLLDEPFGALDAKVRKSLRDSLKDIQREIGITSIL
          *****.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*

sequence1  VTHDPOEALRLSQRIFVLRSPETHQTALSAVILPEGNAPRELHQANLWTLOQQQLLQELGG
sequence2  USTALW (1v8) multiple sequence alignment
          *****.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*.*

```

sequence1 MIKIQQLQHFGSHKVIHNFNLDISKGEIVTFIGKSGCGKSTLLNVIGGGFIHPSSGRVVI
sequence2 KCRORAGYLLVSN---VTDIPVQHRGIGFVFGQYALFRHQTVAQNIA

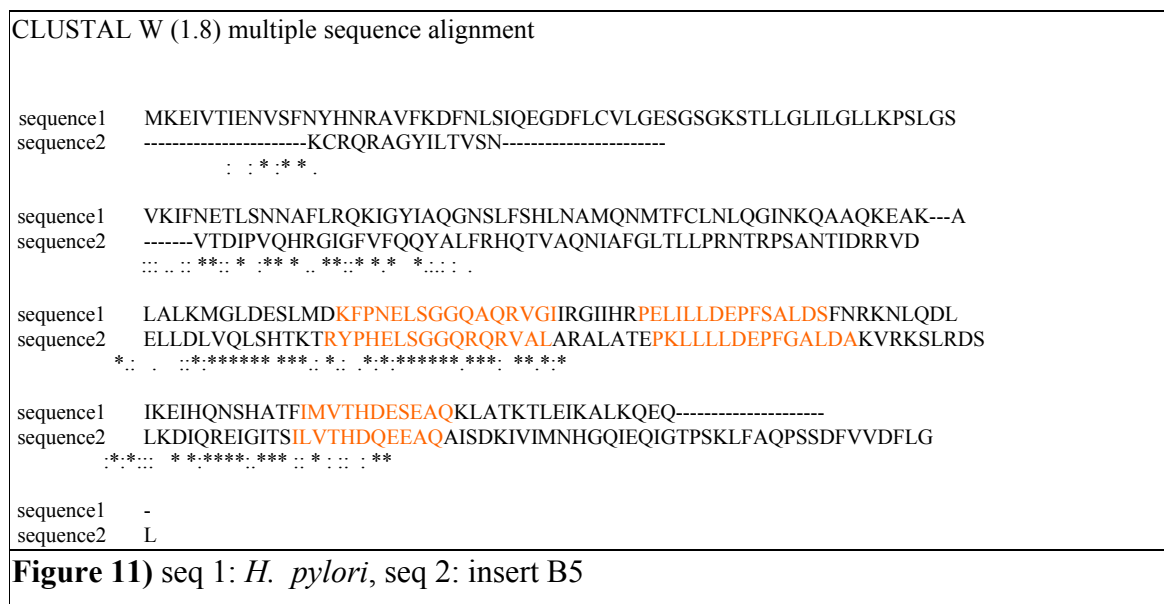
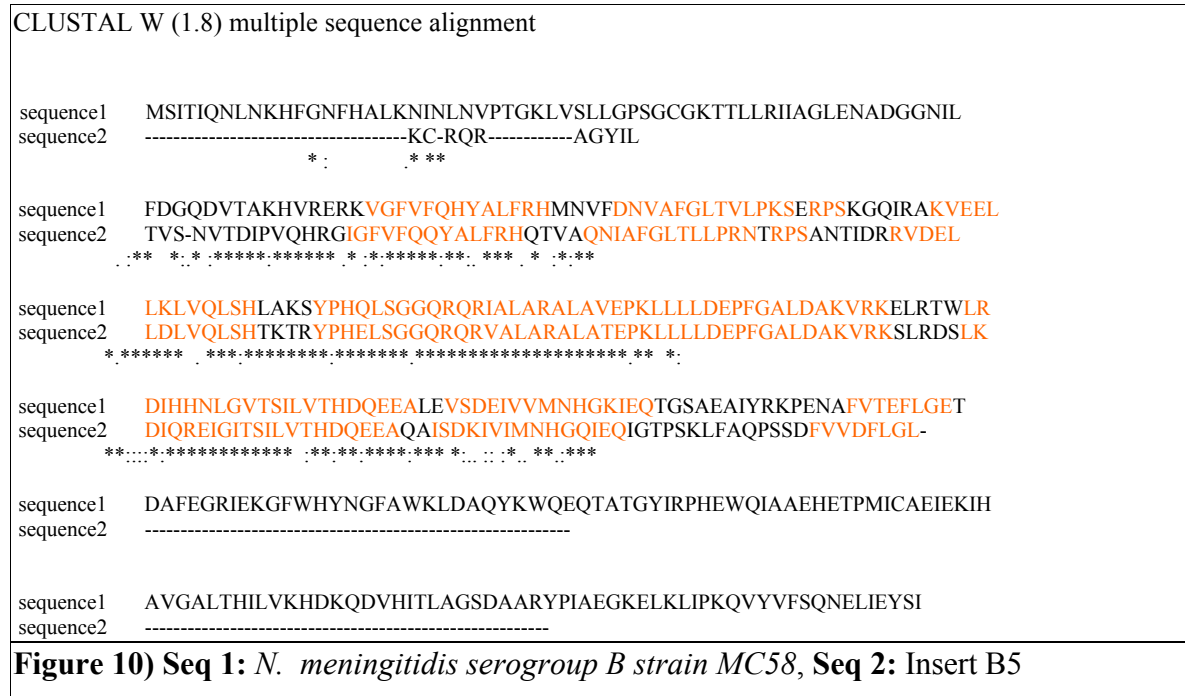
```

sequence1 DNEIKQQSPDCLMLFQHNNLLPWKTINDNIRIGFQQKISDEEINAQLKLVLEDRGKHF
sequence2 FG-----LT----LLPRNTRPS-----ANTIDRRVDELLDLVQLSHTKTRY
      :   ***.*      :  *... *...*...  :
sequence1 PEQLSGGMKQRVALCRAHVHKNPVILMDEPLGALDAFTRYKLQDQLVQLKHKQTSTIILV
sequence2 PHELGGQRQRVALARALATEPKLLLLDEPFGALDAKVRKSLRDSLKDIQREIGITSILV
      *...*** *...*** **  *...*...*...*** *...*...*...  *...***
sequence1 THDIDEAIYLSDRIVLLGEGCNIISQYEITASHPRSRNDSHLLKIRNEIMETFALNHHQV
sequence2 THDQEEAQAIQSDKIVIMNHG-----QIEQIGTPS-----KLFAQPSSDFVVDLGL
      ***.*...*...*** **  *...*...*...*...*...*...*...

```

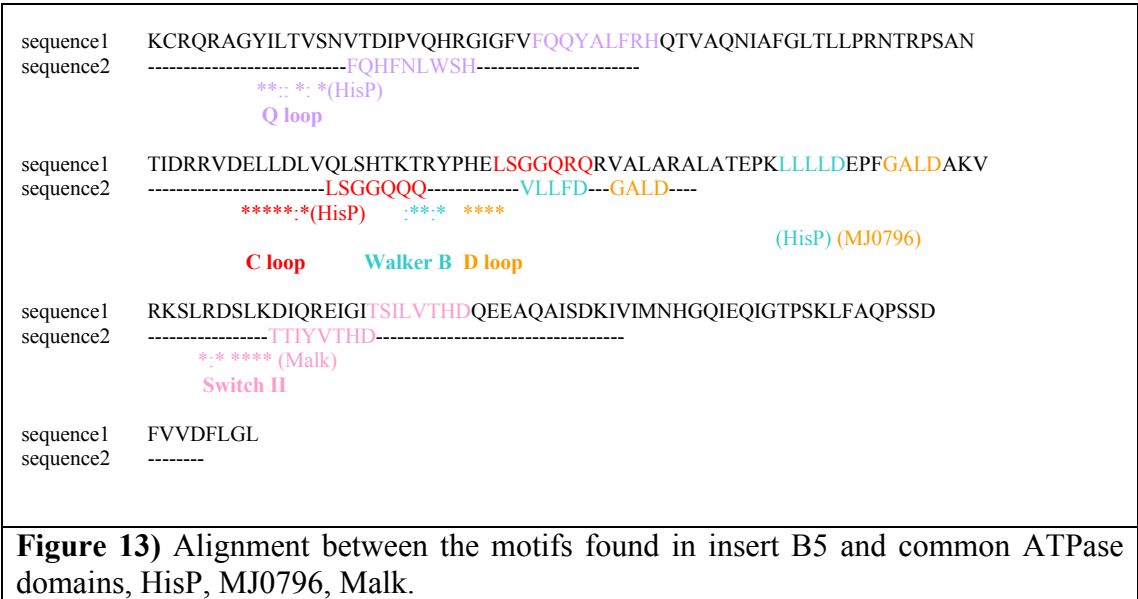
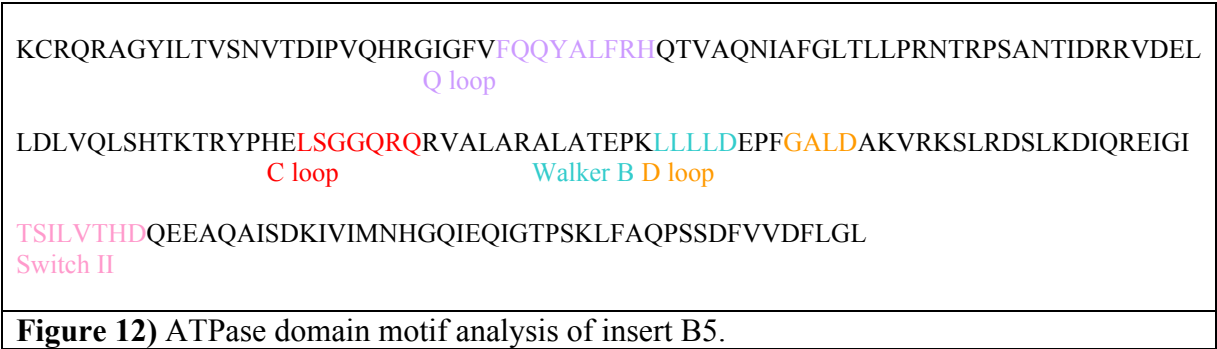
```
sequence1  EPEYYL
sequence2  -----
```

34



3.3.2 Motif search results

The ATPase domain motifs found in insert B5 amino acid sequence (Figure 12). The similarity of them with the motifs of most known, studied ATPase domains, HisP, MJ0796, Malk (Figure13) (Schmitt L 2002).



3.4 PCR results

To get the Walker A motif in the PCR product we did, the expected band range was 700 bp to 1300 bp. After optimization of the PCR done we obtained two separate bands ranging from ~780bp (the lower band) to ~1200bp (the upper band) from the PCR assay prepared by using ABC1 and B5-2 primers (Figure14). These PCR products were cloned in pGEM®-T Easy vector and sequenced. The results of sequencing reactions did not give a significant homology with any motif in ATPase domain of ABC transporter.



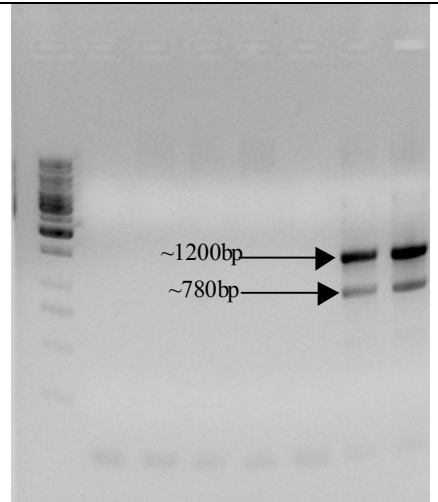


Figure14) PCR assay, forward primer is ABC1. The bands obtained after optimization of the PCR assay.

DISCUSSION

In this study we attempted to sequence the inserts of different clones of *M. catarrhalis* with the intention of identification of some genes in this organism. One of these, clone B5, has significant homology with the ATPase domain of ABC type transporters.

The ATPase domain of an ABC type transporter has conserved motifs: Walker A, Q loop, C loop (signature sequence), Walker B, D loop and Switch II. The ATPase domain is considered as the most conserved domain of the ABC type transporter due to presence of these motifs. The insert B5 has all these except the Walker A motif. Therefore, we speculated that the insert B5 is an ATPase component of a particular ABC type transporter in *M. catarrhalis*. However, the function of this putative ABC type transporter of *M. catarrhalis* is not known. The specificity of ABC type transporter comes from its TMDs not from the NBDs (Schmitt L 2002; Lage H 2003).

In microorganisms, the ABC type transporters are involved in both import and export processes (Dassa E 2001). Also, they are responsible for drug resistance in many bacterial species. For example, ABC type transporters mediate antibiotic resistance of some human pathogens, such as *S. aureus* and *Staphylococcus epidermidis*. The inhibition of ABC type transporter function in these organisms has been a subject of intensive study (Lage H 2003). However, the ABC type transporters, which insert B5 has significant homology with, are generally ion transporters. As a result of this, the putative ABC type transporter of *M. catarrhalis* is not responsible for antibiotic resistance, but most probably is responsible for ion transport. In addition, *M. catarrhalis* is a β -lactamase producer. Since it has such an effective antibiotic resistance mechanism, the probability of having ABC type transporters responsible for drug resistance is very low. In summary, we proposed that the putative ABC type transporter, partially sequenced in this study, is an ion transporter, important for the survival of *M. catarrhalis*. The functional studies of this putative ABC type transporter will be performed after the full sequence of the ATPase domain is completed.

In the insert B5 amino acid sequence there were some stop codons. Actually the presence of these stop codons was an unexpected situation. Generally the stop codons, UAA, UAG, UGA, in universal genetic code may not be stop codons in bacterial genome. For example, *Tetrahymena* uses only one stop codon, UGA, to terminate protein synthesis. The

other stop codons, UAA and UAG are decoded as the glutamine codon (Nakamura Y 1998). In *M. catarrhalis* the stop codon identified so far, is UAA (Du R-P 1998; Kakuda T 2003). However, based on our literature search, which amino acid(s) was coded from the other stop codons, UAA and UAG, in *M. catarrhalis* has not been found yet. Since the nucleotide sequence of insert B5 was translated into its amino acid sequence by a program, which is using universal genetic code, the program might give false stop signals. Another explanation for the extra stop codons in the amino acid sequence of insert B5 could be the following. During clone selection two different fragments from the *M. catarrhalis* genomic DNA library might have come together and inserted into the same vector. If this is the case for clone B5, the stop codons coming before the amino acid sequence consisting of the motifs might belong to the fragment that did not include the ATPase domain. In addition, although we have confirmed the nucleotide sequence of insert B5 by doing the sequencing reaction two times, the polymerase might not work properly and some parts of the sequence could include false bases.

Since the complete sequence of the ATPase domain is not known and the promoter sequence should be found before Walker A motif, the promoter sequence search will be performed after the full sequence of the ATPase component of the putative ABC type transporter in *M. catarrhalis* is completed.

In the insert B5, the length of the ATP-binding cassette domain is 188 amino acids. Our literature search showed that in several bacteria, length of the NBD domain of the ABC transporters is about 215 amino acids (Linton KJ 1998). Therefore, it is necessary to find ~ 27 amino acids to obtain the complete sequence of the ATP-binding domain of the putative ABC transporter of *M. catarrhalis*.

The Walker A is the only motif that was remained to be identified. Since *M. catarrhalis* genome has not been fully sequenced yet, it was not possible to design a specific primer for this. Therefore, we followed another way to complete the putative ATPase domain. The primers were designed from the most conserved amino acid sequence of the Walker A motif of several species, *N. meningitidis*, *P. aeruginosa*, *H. influenzae*, *A. tumefaciens*, and *B. melitensis*. Since the Walker A motifs of different species may show slight variations in some amino acids in the protein sequence (Holland IB 1999), in construction of the primers we took into consideration the most conserved amino acids found in the motif. In addition, in translation of the Walker A into nucleotide sequence we followed two different procedures, considering codon usages of the organisms. In the first approach, we considered the gram negative codon usage and prepared primer ABC1 according to this. As a second approach the ABC2 primer was constructed according to universal codon usage. Finally, similar to the ABC2 the primer ABC3 was designed by using universal codon usage, but in

this case the primer had degeneracy in some bases. The reason for designing of these primers was to include all the possibilities to get a significant band at the end of the PCR assays. In these experiments, the primers, ABC1, ABC2, ABC3 were used as forward primers and primer B5-2 was used as reverse primer. At the end of optimization of the PCR, we obtained two significant bands, ABC1a and ABC1b, 750bp and 1000bp in length respectively. These bands were significant because - based on the predicted length of our ATPase domain - the expected length of the PCR product ranged from 700 bp to 1000 bp. According to this, the bands obtained were promising that they could include the Walker A motif. After isolation of these PCR products from the agarose gel, we directly sequenced the PCR products. However, the direct sequencing of these PCR products did not give a satisfactory result. Then, we made TA cloning of the PCR products and sequenced the inserts. Unfortunately the restriction enzyme analysis, done after isolation of the plasmid, showed that TA cloning was not executed successfully.

Future perspectives

As a future study we will continue to do the sequencing of all clones and complete the nucleotide sequence of these clones. Moreover, we will perform TA cloning and subsequent sequencing reaction of the PCR products.

In order to go to 5' end of the nucleotide sequence, another thing that could be done in future studies is RACE (Rapid Amplification of cDNA Ends). Moreover, the PCR assays could be continued till the Walker A region obtained. After obtaining the complete nucleotide sequence of this ATPase domain, experiments to find its function could be performed. However, since mostly the domains of the ABC type transporters of bacteria were located in different parts of the genome (Schmitt L 2002) prior to the experiments, a careful search of which procedures will be followed should be done.

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www.justbio.com

www.ebi.ac.uk/clustalW

[Align two sequences \(bl2seq\)](#)

www.science-house.org/fungal/learn/Paper.pdf (e-value)

pymol.sourceforge.net/ (The figure in the introduction)

cancer-seqbase.uchicago.edu/primers.html (for primer design information)