Le fonctionnement du transporteur ABC de Streptococcus pneumoniae impliqué dans la résistance contre les peptides antimicrobiens

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THÈSE

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Functioning mechanism of an ABC transporter from *Streptococcus pneumoniae* involved in the resistance towards antimicrobial peptides

Thesis publicly defended on the 28th April 2016
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List of abbreviations:

ABC	ATP-Binding Cassette
AMP	AntiMicrobial Peptide
AUC	Analytical UltraCentrifugation
CAT	Chloramphenicol Acetyl Transferase
CBP	Choline Binding Protein
CDC	Center for Disease Control and prevention
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
Cmc	critical micelle concentration
CYM5	Cymal 5
DDM	*n*-Dodecyl β-D-maltoside
DM	Dodecyl-L-D-maltoside
DHp	Dimerization Histidine Phosphotransfer
dNTP	Deoxyribose containing Nucleoside Triphosphate
FDA	Food and Drug Administration
FOS12	FOS-choline 12
GFP	Green-Fluorescence Protein
GlcNac	N-acetylglucosamine
HK	Histidine Kinase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KANA	Kanamycin resistance cassette
LAPAO	Lauramido-N,N-dimethyl-3-*n*-propylamineoxide
LDAO	Lauryldimethylamine-N-Oxide
MNG3	lauryl Maltose Neopentyl Glycol
lytA	Autolysin A
MATE	Multidrug and Toxin Extrusion
MFS	Major Facilitator Superfamily
MIC	Minimal Inhibitory Concentration
MurNac	N-acetylmuramic acid
NanA	Neuraminidase
NI	Non-Infeceted
NBD	Nucleotide Binding Domain
PALM	Photo-Activated Localization Microscopy
PAOL	Protein Analysis On Line
PavA	Pneumococcal Adhesion and Virulence Factor
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PEP	PhosphoEnolPyruvate
PES	Polyethylsulfone
Ply	Pneumolysin
PmrA	Pneumococcal multidrug resistance protein
OD	Optical Density
OG	Octyl Glucoside
ORF	Open Reading Frame
RR	Response Regulator
SBP	Substrate-Binding Protein
SMR	Small Multidrug Resistance

STORM	Stochastic Optical Reconstruction Microscopy
TCS	Two-component system
THY	Todd-Hewitt broth with 0.5% Yeast extract
TMD	Transmembrane Domain
UDM	*n*-undecyl-β-D-maltopyranoside
UP	Undecaprenyl Phosphate
UPP	Undecaprenyl Pyrophosphate
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
WHO	World Health Organization
WT	Wild Type
YFP	Yellow-Fluorescence Protein

Part 1. Introduction

Introduction – résumé

Streptococcus pneumoniae, aussi appelé le pneumocoque, fait partie des bactéries commensales du nasopharynx humain. Sous certaines conditions, il est capable de devenir pathogène et de causer de multiples maladies comme la pneumonie, la méningite ou des otites. Le pneumocoque est particulièrement dangereux pour les personnes âgées, les enfants et les immunodéprimés. Chaque année, environ un million de personnes meurent à cause d'une infection par le pneumocoque ; la plupart des victimes sont des enfants du tiers monde.

Le pneumocoque est une bactérie fascinante par sa capacité à survivre. Il est capable de s'adapter à de nombreux milieux tels que le nasopharynx, le sang, les articulations ou le cerveau. Souvent, le pneumocoque influence son environnement à ses besoins en générant du peroxyde d'hydrogène et des nombreux peptides et protéines. Ces produits lui permettent de combattre ses compétiteurs dans le même milieu ainsi que d'échapper au système immunitaire de son hôte. De plus, le pneumocoque dispose d'un système efficace d'intégration de l'ADN présent dans son environnement, qui lui permet d'acquérir des gènes lui conférant un avantage sélectif.

Cette propriété joue une rôle très important dans la résistance du pneumocoque aux vaccins et aux antibiotiques. Les deux vaccins existants ciblent la capsule, une structure polysaccharidique entourant le pneumocoque. Cette capsule est essentielle pour la virulence et le sérotype du pneumocoque est determiné par la composition de la capsule. Grâce à la compétence, le pneumocoque peut transformer son ADN et changer la composition de sa capsule ce qui lui permet d'échapper à l'immunisation par le vaccin et éliminer les cibles potentielles des antibiotiques.

La résistance du pneumocoque aux antibiotiques est un problème majeur dans le milieu médical. Le pneumocoque possède de nombreuses ressources additionnelles pour contrer les antibiotiques, par exemple: la mutation les protéines cibles des antibiotiques tel que les protéines liant la pénicilline (PBPs), les systèmes d'expulsions de drogues dont l'extrusion des multi-drogues et toxines (MATE), les pompes de la superfamille des facilitateurs (MFS), la résistance médiée par les pompes (PMR) et de nombreuses protéines de la famille des transporteurs des cassettes ABC.

Les transporteurs ABC sont une ancienne famille de protéines présente dans tous les organismes vivants. Ils peuvent importer ou exporter de nombreux substrats comme des sucres, peptides, toxines, drogues, nutriments, métaux, etc. Pour les bactéries, ils jouent un rôle crucial dans la communication avec l'environnement et la survie. Chez *Escherichia coli*, les transporteurs ABC représentent 5% du génome entier.

Un transporteur ABC typique se trouve dans une membrane lipidique et est composé de quatres domaines. Deux domaines transmembranaires (TMD) et deux domaines responsables de la fonction catalytique (Nucleotide-binding domain ou NBD) d'où vient le nom ATP-binding cassette. Ce domaine est hautement conservé et est composé par des régions Walker A et Walker B, un motif de « signature » LSGGQ, et les boucles H et Q. Le NBD hydrolyse l'ATP induisant le changement de conformation des TMDs qui permet au substrat de passer à travers la membrane. Parfois, les transporteurs ABC sont contrôlés par un système à deux composants (TCS).

Un de ces systèmes d'ABC-TCS dans le pneumocoque (protéines *Spd0804* et *Spd0805* pour l'ABC et *Spd01445* et *Spd1446* pour le TCS), qui est homologue au BceAB-RS chez *Bacillus subtilis*, confère la résistance contre certains antibiotiques appelés les peptides anti-microbiens (AMP). Ce système a été étudié depuis une quinzaine d'années chez *B. subtilis* ou *E. faecalis*, mais le fonctionnement exact reste un mystère. Pendant cette thèse, nous avons utilisé des nombreux outils pour étudier le système *in vivo* dans le pneumocoque, mais aussi *in vitro* par des techniques biochimiques.

1. General Introduction

1.1. General Overview of *Streptococcus pneumoniae*

S. pneumoniae bacterium is a major human pathogen. It is particularly dangerous for young children, the elderly and immuno-compromised persons [7]. According to the World Health Organization (WHO), *S. pneumoniae* kills above 1.6 million people per year out of which over 800,000 are children under the age of five. Center for Disease Control and Prevention (CDC) records over 90 different serotypes of the bacteria based on the polysaccharide capsule. The pneumococcus is a versatile organism, able to cope with a harsh environment. Consequently, the study of its survival mechanisms and toxin resistance mechanisms are of extensive scientific interest.

1.1.1. Discovery of *S. pneumoniae*

Pneumococcus was discovered simultaneously in France and America in 1881. In Paris, France, Louis Pasteur injected rabbits with saliva from a patient that has died from a rabies infection. He observed the rabbit die of an infection different than rabies. He isolated a bacterium from the rabbit's blood and discovered a new bacterial organism less than a 1/1000 of a millimeter in diameter with a number 8 shape (Figure 1.1). He simply called the bacterium pneumococcus [246]. About the same time period, Brigadier General George M. Sternberg, a US Army surgeon, studied malaria in Lousiana, USA. In his studies, he injected rabbits with various samples from local swamps in order to identify the cause of the disease. As a negative control, he chose to use his saliva, which he believed to be sterile. The rabbits injected with this "sterile" control died of either pneumonia or septicemia. The General then isolated a bacterium from the dead rabbit's blood, which he called *micrococcus pasteuris* in reference to Pasteur's discovery a few weeks earlier [246].

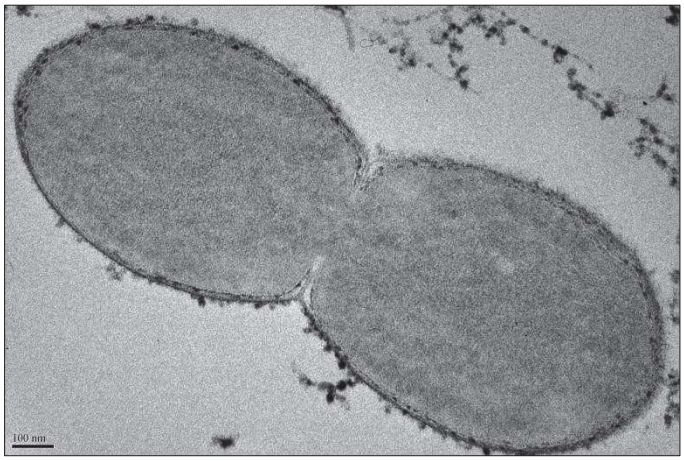


Figure 1.1. *S. pneumoniae* observed on a transmission electron micrograph (TEM). Typical number 8 shape of two berries joined together as seen by Louis Pasteur (photo courtesy of laboratory colleagues).

From 1920 to 1974 the bacterium was known as *Diplococcus pneumonia* [131]. The bacterium got its name from the illness it predominantly causes, pneumonia and the predominant form of two cocci bound together [241]. However, when the bacteria were cultured in liquid broths, chains of bacteria were observed and the genus name changed to *Streptococcus*. It is a name derived from a combination of two Greek words: strepto meaning twisted and kokkus meaning berry [246]. It was also discovered that many humans are healthy carriers of the bacterium and it makes a part of the normal fauna of the nasopharynx. Different strains of both infectious and uninfectious bacteria were discovered. It has been also observed that it can be transmitted through spitting, coughing or sneezing [21].

1.1.2. Diseases and distribution of *S. pneumoniae*

The transition from asymptomatic commensal bacteria to an invasive pathogen largely depends on the balance of the microbial fauna in the nasopharyngeal region. Imbalance may be caused by previous infection, such as influenza [171, 252]. Children, who are just developing their immune system, are the most exposed group of the population towards pneumococcal infection and it represents mortal danger, especially in developing countries [33]. Additionally, the faunal imbalance may cause the pneumococcus to travel to other organs such as: sinuses, lungs, ears, blood, and brain to cause pneumonia, otitis media, meningitis, arthritis, sepsis to name a few (Figure 1.2.) [22, 86, 151]. Interestingly, when a child is ill, it is not uncommon to find *S. pneumoniae* in the ear and simultaneously an *H. influenza*, *M. cattarrhalis*, or *S. aureus* infection in the nasopharynx or vice versa. More rarely, *S. pneumoniae* colonizes both the ear and the nasopharynx [227]. Figure 1.2.

summarizes various diseases caused by *S. pneumoniae* and the pathogenic route of the disease [22, 86, 151].

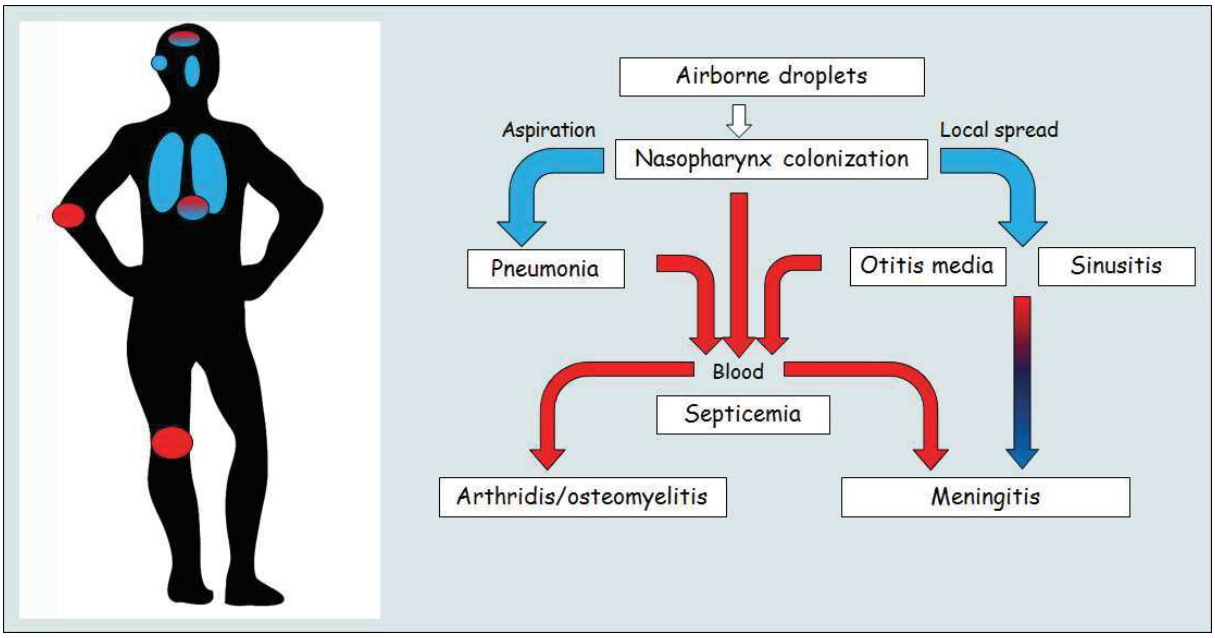


Figure 1.2. Schematic representation of *S. pneumoniae* pathogenic route. Organs and diseases spread through airborne infection are represented in blue. Red organs and arrows represent bloodborne infection (adapted from [21]).

1.1.3. General description of the bacterium

1.1.3.1 Presentation of *S. pneumoniae*

S. pneumoniae is a short bacterium measuring only 1 µm in length which belongs to the phylum Firmicutes, the class Bacilli, the order Lactobacillales, the family Streptococcaceae and the genus *Streptococcus*. This genus includes six major clusters: pyogenic, anginosus, mitis, salivarius, bovis and mutans. The mitis cluster, also known as the oral streptococcal cluster, includes *S. mitis, S. oralis, S. cristatus, S. infantis, S. peroris,* and *S. pneumoniae.* The pneumococcus is an anaerobic, but aero-tolerant Gram positive bacterium. It does not form spores neither any flagellum, but it possesses pili. A typical feature of the pneumococcus is a polysaccharide capsule surrounding its surface.

Unlike other streptococci, *S. pneumoniae* oxidizes hemoglobin and causes alpha-hemolysis. This means, the pneumococcus is easily distinguishable when grown on blood agar by a green/brown halo around colonies. Other characteristic aspects include bile solubility, inulin hydrolysis and optochin sensitivity. Even though these simple tests do not

ensure 100% accuracy in *S. pneumoniae* identification, they remain the standard operating procedure in clinical identification of a pneumococcal infection [22, 186]. In more difficult cases, or in cases where further identification is required, PCR amplification of virulence genes autolysin (*lytA*) and pneumolysin (*ply*) is performed routinely [21, 22].

1.1.3.2. Cell wall synthesis

The function of the cell wall is to protect the bacterium from bursting open due to internal turgor pressure and also to determine the final shape of the bacteria. This cell wall is composed of peptidoglycan (PG), which forms a chain and eventually a mesh into which teichoic acid (TA) may be incorporated. The peptidoglycan composition is specific for each species of bacteria and together with hydrolases determines the shape and geometry of the peptidoglycan chain [164, 237].

Peptidoglycan synthesis is a difficult, yet vital process involving more than 20 enzymes in various parts of the cell: cytoplasm, membrane and cell wall. Peptidoglycan begins to be synthesized first in the cytoplasm of the bacteria before being attached to a lipid carrier in the membrane, forming the lipid II precursor. Lipid II is then flipped to the exterior and the peptidoglycan is added into the cell wall [87, 257].

Figure 1.3. shows in detail the cell wall formation: the first peptidoglycan precursor, UDP-N-acetylglucosamine (UDP-GlcNac), must be converted to UDP-N-acetylmuramic acid (UDP-MurNac) by the addition of a lactyl group to the glucosamine. Then five residues are successively added to the UDP-MurNac by several ATP-dependent ligases. Sequentially, the MurNac-pentapeptide is added to undecaprenyl phosphate (UP) to form undecaprenyl-pyrophosphoryl-Mur*N*ac-pentapeptide also known as lipid I. MurG protein then adds GlcNac to the lipid I forming lipid II (undecaprenyl-pyrophosphoryl-MurNAc-(pentapeptide)-GlcNac) [164, 186]. The last steps before flipping the lipid II to the external milieu is the amidation of D-Glu in position two of the pentapetide and adding a branch of two residues to the Lysine in position three [87, 186].

Once lipid II has been synthesized, flippases RodA and FtsW flip it through the membrane to the external milieu. On the outside of the cell two classes of penicillin binding proteins (PBP) usually take charge of the lipid II. Class A PBPs elongate the peptidoglycan chain by trans-peptidation and they cross-link the chains by trans-glycosylation and the class B PBPs only perform trans-peptidation [87, 257]. Since the cell wall synthesis is essential for cell survival and division, it is frequently a target of various drugs and antibiotics [257].

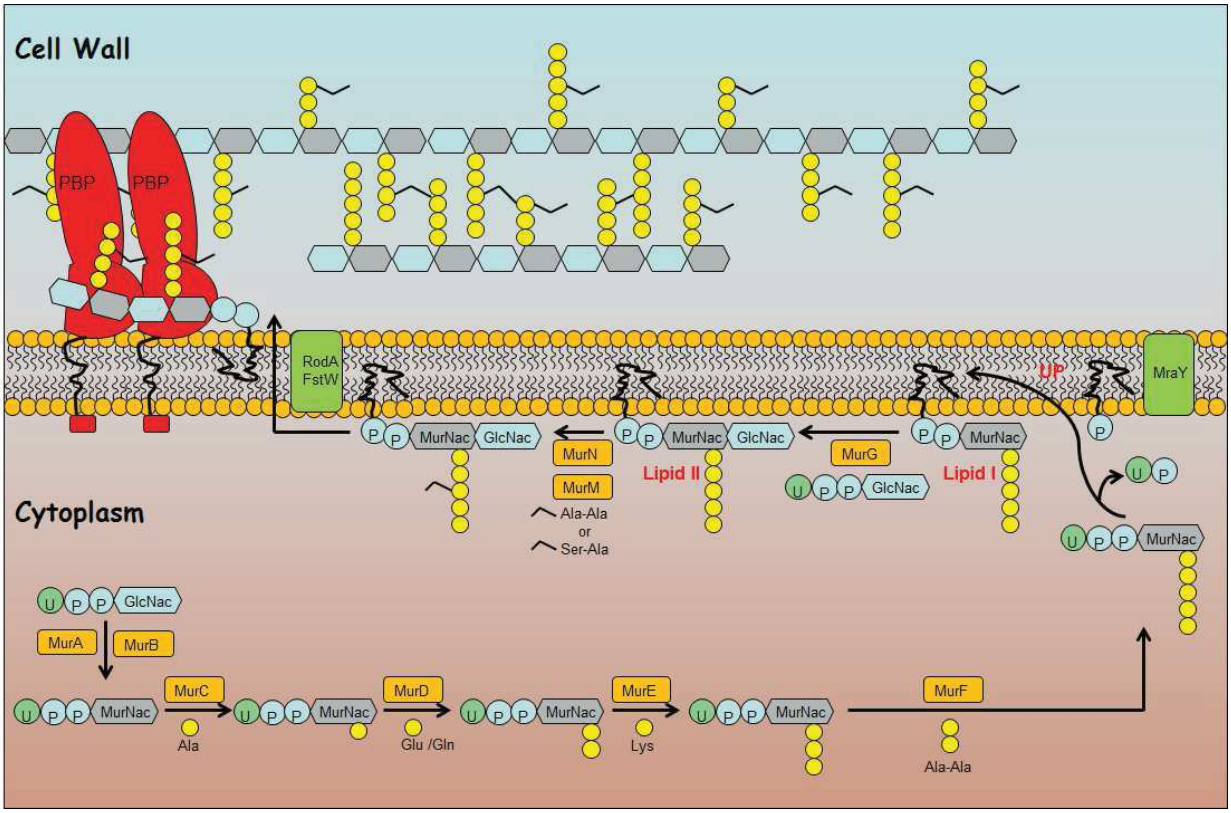


Figure 1.3. ***S. pneumoniae*** **peptidoglycan synthesis.** N-acetylmuramic acid-pentapetide (MurNAc-pentapetide), a lipid II precursor, is synthesized in the cell and attached to undecaprenyl phosphate (UP) to form lipid I. Lipid I matures through addition of N-acetylglucosamine (GlcNac) and becomes lipid II, which is consequently flipped to the external milieu. Names of the enzymes involved in each step are in yellow boxes. The peptidoglycan is then added to the chain through penicillin binding proteins (PBP) activity [186].

1.1.3.3. Colonization

The upper respiratory tract presents a good niche for many bacterial species. During the first few months of human life a normal fauna establishes itself in the nasopharynx. The colonization by *S. pneumoniae* occurs through horizontal dissemination, spreading of potential pathogens between individuals [22]. The host immunity, as well as the fauna itself, plays a regulatory role for bacterial populations in the niche.

For successful colonization, the pneumococcus needs several adhesion systems as shown in Figure 1.4. to attach itself to the host surface, which usually are epithelial cells. Adherence proteins facilitate attachment through specific interactions as well as non-specific physiochemical interactions by changing the hydrophobic and electrostatic properties of the bacterial cell surface [21,104, 226]. There are many adhesion proteins, which play important roles in different stages of colonization. Some examples of these adhesion proteins include

Pneumococcal adhesion and virulence factor A (PavA) and choline binding proteins (CBP) [90, 118].

A crucial tool for *S. pneumoniae* to colonize its niche, is its polysaccharide capsule. This capsule, normally 200-400 nm thick, surrounds the peptidoglycan cell wall. It is interesting to note that during nasopharyngeal colonization, the capsule narrows down and thickens upon invasive infection [211]. The bacterial strains are determined by their capsule or, in some extent, the lack of it, since there have been a few rare cases of infections by unencapsulated *S. pneumoniae* strains [47]. The *S. pneumoniae* capsule is multifunctional as it protects the bacterium from phagocytosis, complement cascade, specific antibodies and some antibiotics, as well as it prevents aggregation [74, 106, 117, 148, 167]. Moreover, it has also been demonstrated in the past that *S. pneumoniae* has the ability to switch its capsule composition and therefore its serotype. This creates some concerns for the use and production of vaccines as the bacteria can evade the specific antibodies by switching serotype and re-colonize the same individual [254]. Capsule switching is made possible thanks to a high level of competency, which enables the pneumococcus to take up DNA from the environment and to transform its genome. It has been shown as early as in 1928 that avirulent strains became virulent if they were incubated for some time with dead virulent strains and then injected into a rabbit [91]. Nowadays there are only two commercially available vaccine types based on this polysaccharide capsule, which protect only against about a third [2, 160] of the total over 90 pneumococcal serotypes [253].

Under the right conditions, the pneumococcus may colonize a number of organisms other than humans. Animal experimentation is an essential tool in studying infectious diseases. Typical models used to study *S. pneumoniae* virulence include mice, rats and rabbits to test drug effects as well as genetic modifications of the bacterium [34]. Another model, which is much more economically and ethically interesting is the *Drosophila melanogaster* model. The drosophilae rather short life span and relatively easy upkeep make it an ideal platform for primary infection studies and examine the streptococcal global virulence. It is impossible to explore the colonization and invasion processes [185].

Some avirulent strains of the *S. pneumoniae* have been discovered [47, 98]. These strains lack the polysaccharide capsule, such as the modified from the D39 strain, R6, which is a good model to work with in the laboratory, since it allows researchers to work in a Level 1 biosafety laboratory. In this study, we work with a virulent D39 strain, which requires Level 2 biosafety equipment.

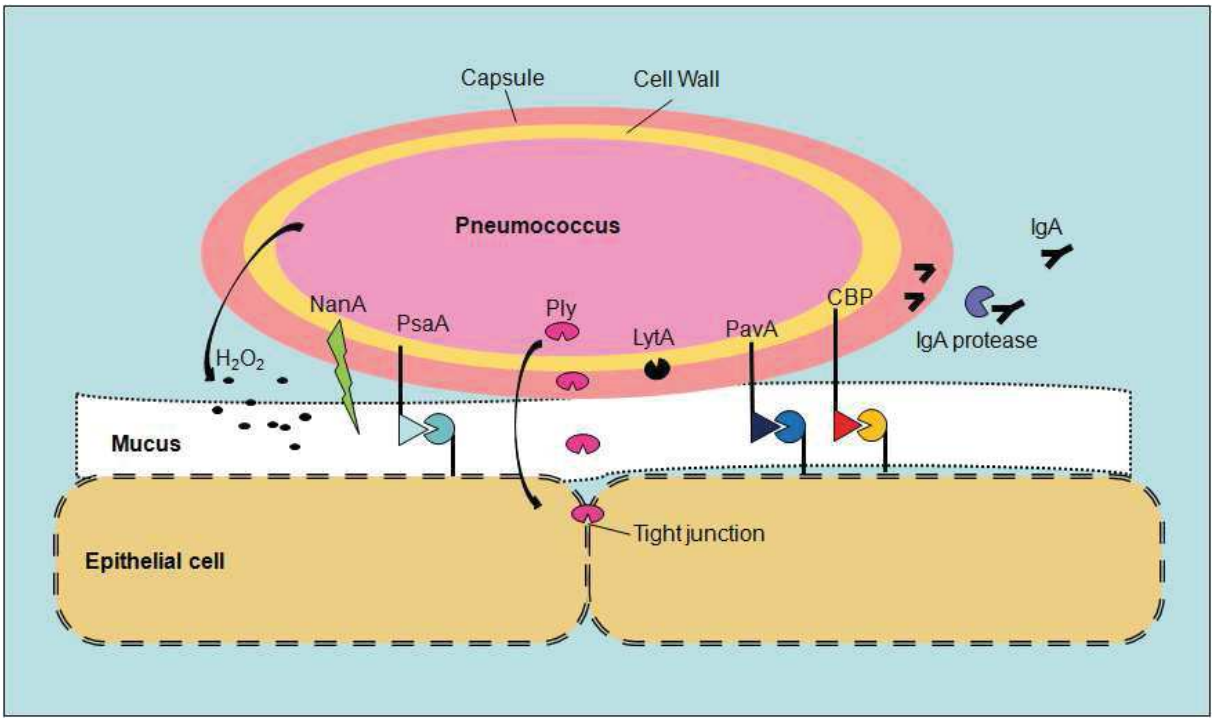


Figure 1.4. Schematic representation of *S. pneumoniae* virulence factors and their effect. A number of virulence factors are necessary for the viability of the pneumococcus. Several adhesion proteins are required for the bacteria to be able to survive the harsh environment of human nasopharynx. Free 'pacmen' are shown together with their targets. 'Pacmen' attached to the epithelial cells are the receptors of pneumococcal adherence proteins represented by a triangle attached to the pneumococcal cell wall. NanA flash simulates its action to weaken the mucus layer. Cleaved IgA pieces bind to the capsule preventing functional IgA to bind to the bacterium [21].

1.1.3.4. *S. pneumoniae* interactions with its environment

1.1.3.4.1. *S. pneumoniae* changes its environment

The survival of the *S. pneumoniae* depends on the interaction with its immediate environment and correctly responding to stimuli. To react to these incentives a combination of many factors is necessary. These factors are also sometimes main contributors to virulence towards the host, but they also play a role in interacting with other inhabitants of the niche. Pneumococcus has been found sharing the biofilms covering children's nasopharynx together with *Haemophilus influenza, Moraxella catarrhalis, Staphylococcus aureus, Staphylococcus epidermidis* to name a few [166, 184]. Biofilm formation depends also on the function of a cell surface protein neuraminidase (NanA) [21]. By its metabolism, the pneumococcus naturally releases hydrogen peroxide, as a way to control competition, and together with NanA to reduce mucus viscosity and to prepare epithelial surface for colonization [21, 205]. Unlike other bacteria, the pneumococcus is quite resistant to hydrogen peroxide and its

release favors *S. pneumoniae* proliferation in expense of the others [118]. Over time however, it may become toxic to the pneumococcus itself [118].

Another factor with which *S. pneumoniae* changes its environment is the LytA protein, which is an important component of the pneumococcal virulence [16]. LytA is involved in autolysis as well as fratricide and penicillin resistance. In fact, it disintegrates the bacterial cell wall, which causes the cells to lyse releasing other proteins, peptides and teichoic acids, which add to virulence and favor pneumococcal growth [103, 118, 161]. A prime example is the release of pneumolysin which has a number of functions including: opening of tight junctions between epithelial cells, including alveolar junctions [197, 205], inhibition of ciliary beat in respiratory tract [73], erythrocyte lysis [163], complement pathway activation [25], inhibition of antibody production [75].

Other factors are also essential to counter the host immunity. Factor IgA protease illustrates well this function as it cleaves IgA into two fragments [21]. Therefore, the immunoglobin is unable to bind and opsonize the pneumococcal cell. Furthermore, one of the subunits of the cleaved antibody then enhances the adherence of the bacteria to epithelial cells. As a result, IgA proteases serve in both immune system evasion and cell adhesion and colonization [189, 192, 247]. Figure 1.4. illustrates the actions of different proteins in their interaction the nasopharyngeal environment.

1.1.3.4.2. *S. pneumoniae* responds to its environment

Human nasopharyngeal region is a harsh environment presented not only by the mucus and the epithelium, but also by other host defenses such as antimicrobial peptides (AMP) expression. Competing rivals, like the pneumococcus, also express AMPs and other toxins in order to hinder pneumococcal proliferation. Besides, most of the bacteria in the nasopharyngeal region belong to the Firmicutes phylum known to produce an enormous number of AMPs [225], which are discussed in greater detail in section 1.2. The pneumococcus counters these toxins by a combination of factors described in section 1.1.4.

Many survival and virulence factors display some sort of redundancy, such as pneumococcal surface adhesion protein (PsaA), which fulfills many vital functions in complexes with othe proteins. Its deletion greatly attenuates the pneumococcal virulence. Other than adhesion, PsaA has additional roles including manganese transport and oxidative stress resistance [65, 115]. Another example of redundancy is having several proteins transporting the same substrate, which is of a great advantage to the bacterium. For instance, deletion of either pneumococcal iron acquisition A (PiaA) or pneumococcal iron uptake (Piu)

causes a slight attenuation of bacterial growth, but deletion of both transporters is lethal [28, 115]. A third kind of redundancy is having promiscuous transporters; a single protein may bind and sometimes transport several substrates with different affinities and therefore at least partially replace the function of another protein. For instance, PsaA can also transport zinc, and partially replace the loss of function of the principal zinc importer Adc [65]. All of these abovementioned proteins belong to the ATP-binding cassette (ABC) protein superfamily.
Proteins belonging to this superfamily are heavily involved in interaction with the environment and cell survival. ABC transporters are responsible for nutrient uptake, including sugars, lipids, minerals and they also export materials such as toxic waste. About 60 ABC transporters have been identified in *S. pneumoniae* regardless of the strain. Roughly 60% of them are importers, the rest are either exporters or have another function [65]. As described above, ABC transport systems are extremely versatile and are crucial for survival and are discussed in greater detail in section 1.3. of the introduction.

Another important player in the interaction with the environment of the pneumococcus are two-component systems (TCS). *S. pneumoniae* genome codes for thirteen different TCSs [182, 229]. A TCS is typically composed of a histidine kinase (HK) and a response regulator (RR). The pnemococcal genomal additionally contains a single "orphan" RR [229]. Habitually, TCS systems are gene regulators, which sense substrates and mount an adequate response to the stimulus. Universally speaking, many TCSs strictly regulate a single target protein, but there are also more versatile systems, such as bacteriocin-like peptide, TCS13, which regulates sixteen different genes [159, 182, 229, 232]. All pneumococcal TCSs have overlapping genes, where HK overlaps the RR, suggesting that they get transcribed as a single unit. Of the thirteen TCSs, only seven have been studied in various degree of detail, and five of them are known to regulate ABC transporters. TCS systems are discussed in greater detail in section 1.4. of the introduction.

1.1.4. Antibiotic resistance

As mentioned above, the pneumococcus is surrounded by AMPs along with other drugs and toxins and therefore requires the presence of resistance mechanisms giving bacteria the edge to survive in the hostile environment. Thanks to this selective pressure, *S. pneumoniae* has acquired a number of antibiotic resistances to various drugs which is becoming a serious health concern in both developed and developing countries. The paradox is that the majority of antibiotic resistant infections are acquired at hospitals [61, 235].

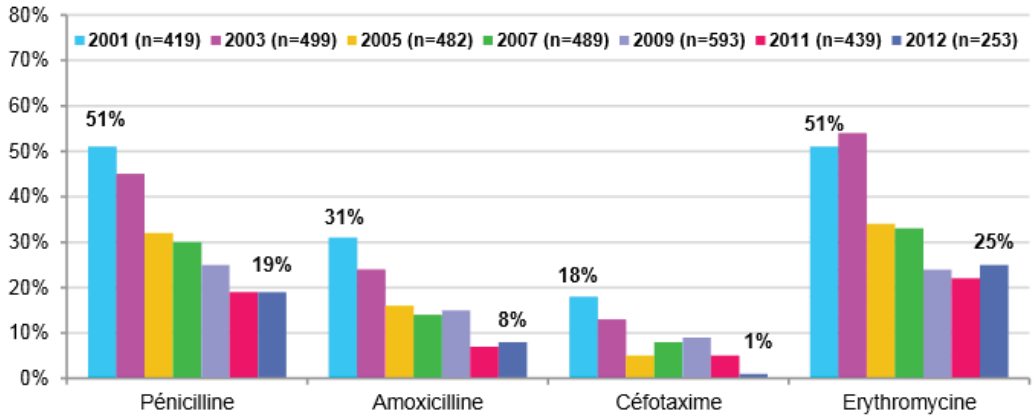


Figure 1.5. Evolution of pneumococcal antibiotic resistant infections in children in France between 2001 and 2012. Y-axis shows the percentage of resistant strains towards given antibiotic. A number of strains display resistance towards several antibiotics. A clear negative trend of occurrence of antibiotic resistant pneumococcal infection is apparent (figure taken from [240]).

Both the WHO and the CDC warn of the increasing number of antibiotic resistant strains. First clinically significant case of penicillin resistant *S. pneumoniae* infection has been reported as early as in 1967 in Guinea [96]. Afterwards, resistant strains have appeared around the world through misuse and overuse of antibiotics. Surprisingly, in some parts of the world, like Australia, the first case of antibiotic resistant pneumococcus has only been discovered in 1996 [42]. In other countries, such as France the trend of antibiotic resistant strains has been at a dangerous rise throughout the 1980's and 1990's. The French government intervened by limiting antibiotic use and introducing a vaccination program, which successfully diminished the cases of antibiotic resistant pneumococcal infections (Figure 1.5.). In the early 2000s 51% of pneumococcus infection cases in children were penicillin resistant, while "only" 19% were resistant in 2012 [240].

A common resistance strategy to β-lactam antibiotics (penicillin) is the modification of the target. Penicillin resistant pneumococci have mutated PBPs, which reduces the affinity of the drug to its target. Resistant strains then require up to 400 times higher antibiotic concentration to be killed [31]. In addition to low affinity PBPs, the *S. pneumoniae* may upregulate its expression of phosphate ABC transporter Pst. This ABC transporter is an influx pump and even though its exact role in the resistance process is unknown, it has been shown to increase β-lactam resistance even further [69]. Besides, the Pst complex is also responsible for metal uptake [179]. In mouse models, the deletion of parts of the complex caused attenuation of virulence of the bacteria [65, 179].

Other antibiotic resistance mechanisms in the pneumococcus include three multidrug and toxin extrusion (MATE) efflux pumps [230], one major facilitator superfamily (MFS)

transporter, the pump-mediated resistance (PmrA) [187], and some ABC transporters [65]. Of the three MATE proteins, only one, DinF has been studied in detail. It confers resistance in particular to the antibiotics quinolone and moxifloxacin, and partial resistance to ciprofloxacin and levofloxacin [230]. *S. pneumoniae* strains resistant to norfloxacin have been shown to display a high activity of the PmrA efflux transporter [187]. It is clear though that these efflux transporters do not work alone. More studies have to be conducted to fully understand drug resistance in the pneumococcus [9, 183, 187, 230].

There are several ABC transporters conferring resistance to drugs, antibiotics and AMPs. One of the most well-known MDR ABC transporters in the pneumococcus is the PatA/PatB antibiotic transporter a heterodimeric ABC transporter [9]. The deletion of either PatA, PatB or both causes severe susceptibility to ethidium bromide, acriflavine, ciprofloxacin, and norfloxacin compared to the wild type pneumococcus [9, 24]. It has been also suggested that PatA alone may form a homodimer conferring reserpine resistance, but this has been ruled out by the purification and biochemical characterization of the whole transporter, PatA/PatB or its individual subunits [24]. PatA may also be a promiscuous half-transporter which works with several partners to transport various substrates [9, 24, 82, 152].
LL-37 and erythromycin (human AMPs) resistance is conferred by the mefE/mel ABC transporter. The MIC for most strains is ~15 μg/ml which is a quite high concentration [150]. Mutants lacking the activity of this ABC transporter cannot effectively colonize humans. The protein has not been studied in much detail yet [135, 150, 255].

Upon colonization of a niche, the pneumococcus begins expressing AMPs of its own. An ABC transporter BlpAB exports the accumulating peptides from the cytoplasm to the exterior. There is a wide range of bacteriocins that are effective contra-species as well as contra-strain. The export is regulated be a TCS similar to comDE described further in the introduction in section 1.5. [33, 54, 147].

1.2. Antimicrobial peptides (AMP)

Since the 1920's AMPs have aroused interest in the scientific community. In literature, antimicrobial peptides are often classified as antibiotics and the terms are sometimes used interchangeably. Their potential lies in the fact that although they have been discovered some time ago, their use has been more sporadic and more isolated than standard antibiotics [5]. Generally speaking, because bacteria have not been exposed to AMPs on such enormous scale as to antibiotics, they have not been able to develop high resistance to them. Though AMPs are found in the natural environment, their concentration is relatively low [23]. Medical treatment introduces much higher AMP concentrations towards which the bacteria should not be resistant. In addition, bacteria inhabiting humans likely did not encounter AMPs found in plants or fungi, which are being considered as attractive alternative therapeutic agents. Therefore it is crucial to study their interaction with bacteria to prevent rapid resistance towards these compounds. Therefore it is necessary to assess the rentability of using AMPs as an antibiotic alternative [5].

1.2.1. General introduction of AMPs

AMPs are an inseparable part of the bacterial environment. Humans carry on average about two kilograms of bacterial commensal fauna [37], therefore competition between bacteria is fierce. They employ AMPs as way to outcompete their rivals. Additionally, the host expresses its own AMPs to limit microbiota proliferation. The potency and targets of AMPs vary, not all are bactericidal, but 'merely' bacteriostatic. Nevertheless, even bacteriostatic AMPs give a competitive edge to the bacteria producing them. Other bacterial AMPs may be directed against the host, which prevents it to mount adequate immune response [23].

AMPs are relatively small peptides no longer than 50 amino-acids. Their structure can be either linear, circular, α-helices or β-sheets. They have been shown to be sometimes selective against either Gram positive bacteria, Gram negative bacteria, fungi, or Protista [27, 190]. Remarkably, AMPs are produced by all known organisms (probably with the exception of viruses). Strikingly, the majority of AMPs are cationic. The common opinion is that the positive charge of AMPs is necessary to be attracted by the negatively-charged bacterial membranes [110, 219]. Another one of the amazing characteristics of these molecules is their potency at extremely low concentrations (nM-μM range) [44].

AMPs have been used in various applications such as food preservatives (nisin) [40], or in the medical field as an alternative to classical antibiotics (vancomycin) [168]. Even so, the full potential of these molecules has not been exploited yet [44, 119, 219]. Their potential also lies in numerous modes of action ranging from: membrane pore formation, interference with cell wall synthesis, septum synthesis, inhibition of enzymatic activity, or tampering with DNA/RNA [5, 27]. Many of these AMPs are exported by ABC transporters which work as self-protection or protection from AMPs produced by another species.

1.2.2. AMP classes

AMPs are classified into different groups according to their size, structure, charge, and many other aspects. To date, more than 2000 natural AMPs have been identified [219, 243], but there is no standardized classification system. Table 1 presents basic AMP classification suited for the purposes of this work. Each AMPs used in this work will be presented in more detail further in the chapter.

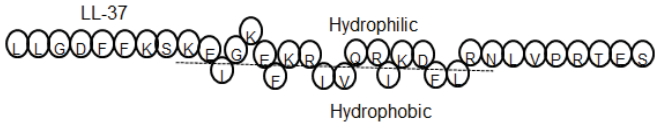
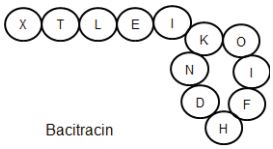
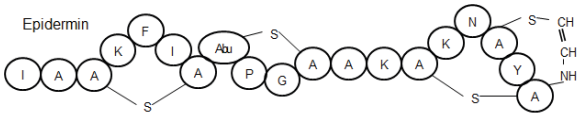
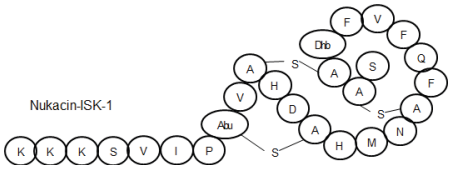
Class	Source	Example	Structure
Defensins	Mammals Insects	LL-37, CRAMP-I, CAP-18	
Non-ribosomal Bacteriocins	Bacteria	Bacitracin Vancomycin	
Ribosomal Bacteriocins	Gram+ Bacteria		
Class I		Nisin A Epidermin Mercasicin	
Class II		Lactococcin Nukacin-ISK-1	

Table 1.1. Classification of antimicrobial peptides

Defensins constitute an important class of AMPs. These molecules are expressed by eukaryotic cells and represent an imperative part of the innate immunity, which protects the organisms from primary infections. Plants and insects lack humoral immunity and as a consequence, their innate immunity is very strong mainly due to the immense variety of defensins they produce. As a result, they are being closely studied as possible therapeutical agents [219, 243]. In mammals, the cells which produce a considerable variety of defensins are epithelial cells and leukocytes. These peptides display an immense variety, and are split into three different classes with little common motifs shared between them [5, 58, 64, 149, 256]. A well-studied example of a human defensin is LL-37, which is active not only against bacteria, but other disease-causing agents (fungi, viruses) as well.

Bacteriocins are AMPs produced by bacteria, primarily by Gram-positive species, but some may be active against a few Gram-negative bacteria as well [125]. It has been shown in various studies that the phylum Firmicutes and especially the genus *Streptococcus* produce an abundant variety of bacteriocins [60]. Numerous peptides are post-translationally modified; their mRNAs are often disproportionally longer than the resulting peptide [110, 111].

Non-ribosomal AMPs often include non-proteinogenic amino-acids. There are a number of other chaperones called peptide synthases that alter the peptide by methylation, cyclization, acylation, glycosylation and/or addition of other amino-acids. As a result, these AMPs sometimes vary even in the same bacteria producing them [76]. These modifications may change the target or binding affinity. Nevertheless, they contain rarely more than 20 amino-acid residues and are often cyclic [37]. Non-ribosomal AMPs include bacitractin, vancomycine and ramoplanin. In the literature, they may to be classed as bacteriocins in some publications and as antibiotics in others [37, 76, 128]. For the purposes of the thesis, they belong to the bacteriocin group, since they are produced primarily by bacteria and their targets are bacteria.

Class I bacteriocins are lantibiotics (referring to lanthionine-containing antibiotic peptides) and they represent a class of bacteriocins which are post-translationally modified by the formation of thioether bridges between unusual amino acids such as lanthionine, methyllanthionine, D-alanine etc. These bridges provide stability and inhibit the action of peptide-digesting enzymes [18]. Their characteristic is hydrophobicity and they are slightly elongated compared to other AMPs. As a result, they are pluripotent and can have more than one mode of action [44, 110, 111]. Another advantage of lantibiotics is that slight mutation in amino-acid composition may widen their active pH range, change membrane binding target, improve stability or enhance cell wall binding [110]. In the past, lantibiotics were being categorized into different groups, but the grouping proved to be artificial and inefficient. Lantibiotics include nisin, duramycin, epidermin, Pep5 and others.

1.2.3. AMP modes of action

As mentioned above, many of the studied antimicrobial peptides inhibit cell wall formation at the lipid II carrier. Lipid II is an essential player in bacterial cell wall formation as described previously in the introduction in section 1.1.3.1. In a lipid raft, the lipid II translocates peptidoglycan across the cell membrane using a flippase transporter and then enables the peptidoglycan polymerization through trans-peptidation and trans-glycosylation [164]. On the cell surface, lipid II releases peptidoglycan. Undecaprenyl pyrophosphate

(UPP), the peptidoglycan carrier, is dephosphorylated to UP in the outer membrane in order to be flipped into the cytoplasmic side of the bacterial membrane. UP is afterwards re-phosphorylated again and re-charged with peptidoglycan. It then flips again to the outer membrane. The lipid II cycle is illustrated schematically in Figure 1.6., including intervening AMPs; some prevent the release of peptidoglycan, some inhibit trans-peptidation and trans-glycosylation, others may inhibit UPP-UP phosphorylation, and some even form pores in the cell membrane [13, 125, 190].

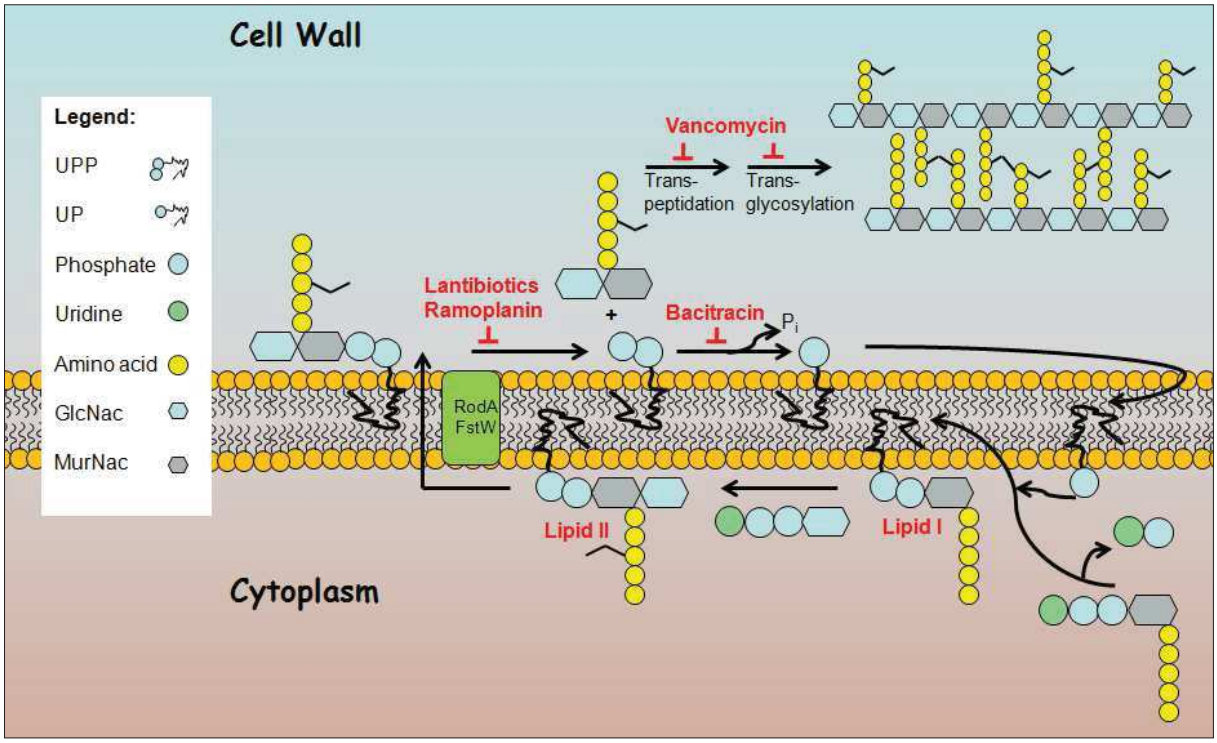


Figure 1.6. Schematic representation of peptidoglycan biosynthesis and its inhibition by AMPs. Important steps in cell wall biosynthesis are depicted, and their cellular location is indicated on the left. Amino acids are symbolized by small yellow circles. Lipid II consists of the MurNac/GlcNac-pentapeptide building block, covalently linked to the lipid carrier molecule UP via a pyrophosphate ester bridge. The steps of cell envelope biosynthesis linked to UP are referred to as 'lipid II cycle'. Chosen examples of AMPs are placed next to the step of the cell wall formation they inhibit [200].

1.2.4. AMPs used in this study

Bacitracin is a bacteriocin which binds to UPP and prevents its dephosphorylation and translocation from the cytosol to the outer leaflet of the membrane as seen in Figure 1.6. As a result, bacitracin inhibits the formation of lipid I [222, 223]. Likewise, bacitracin can also bind various metals, depleting available nutrients available to the bacteria [68, 222]. It is a potent AMP, which is active at low concentrations (μM) [125]. It is synthesized by *B.*

lichenirormis and a few strains of *B. subtilis* [6, 108, 114]. As bacitracin is used very widely in livestock feed, it is crucial to understand the mode of action of proteins providing bacterial resistance towards it. The American Food and Drug Administration (FDA) also approved bacitracin use in humans, but its concentration in blood must be closely followed [125]. Lately, bacitracin structure has become the scaffold upon which scientists have been trying to synthesize new therapeutical agents [68].

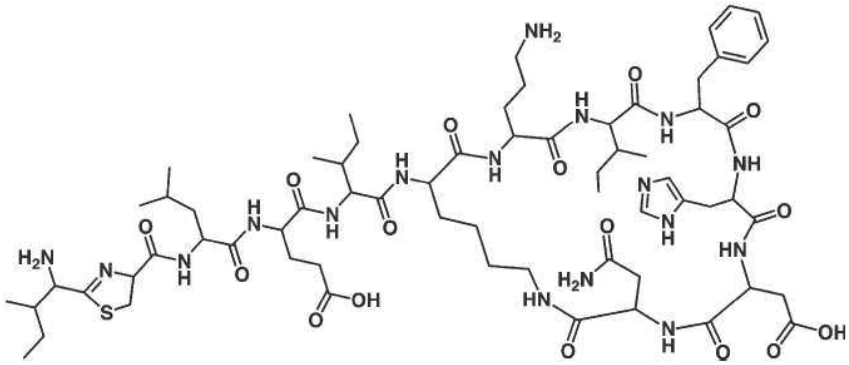


Figure 1.7. The structure of bacitracin [68].

Nisin is a lantibiotic first isolated from *S. lactis* in 1928 [15, 92]. Nisin is active against almost all Gram-positive bacteria even at nanomolar concentrations and against some Gram-negative bacteria at higher concentrations [181]. It is widely used as a food preservative, as it has a broad range of activity and very low toxicity to humans [3, 119, 157]. At high concentrations, nisin binds indiscriminately to bacterial membranes and through interaction of its hydrophobic domains incorporates into the membrane. Through this action it creates relatively large pores (Figure 1.8.B) and kills the bacterium by "leaking." At low concentrations, nisin operates by binding to the lipid II and its derivatives and by changing its conformation it blocks the peptidoglycan at the UPP and hence it inhibits formation of the cell wall (Figure1.8.C) [92, 97, 99, 238].

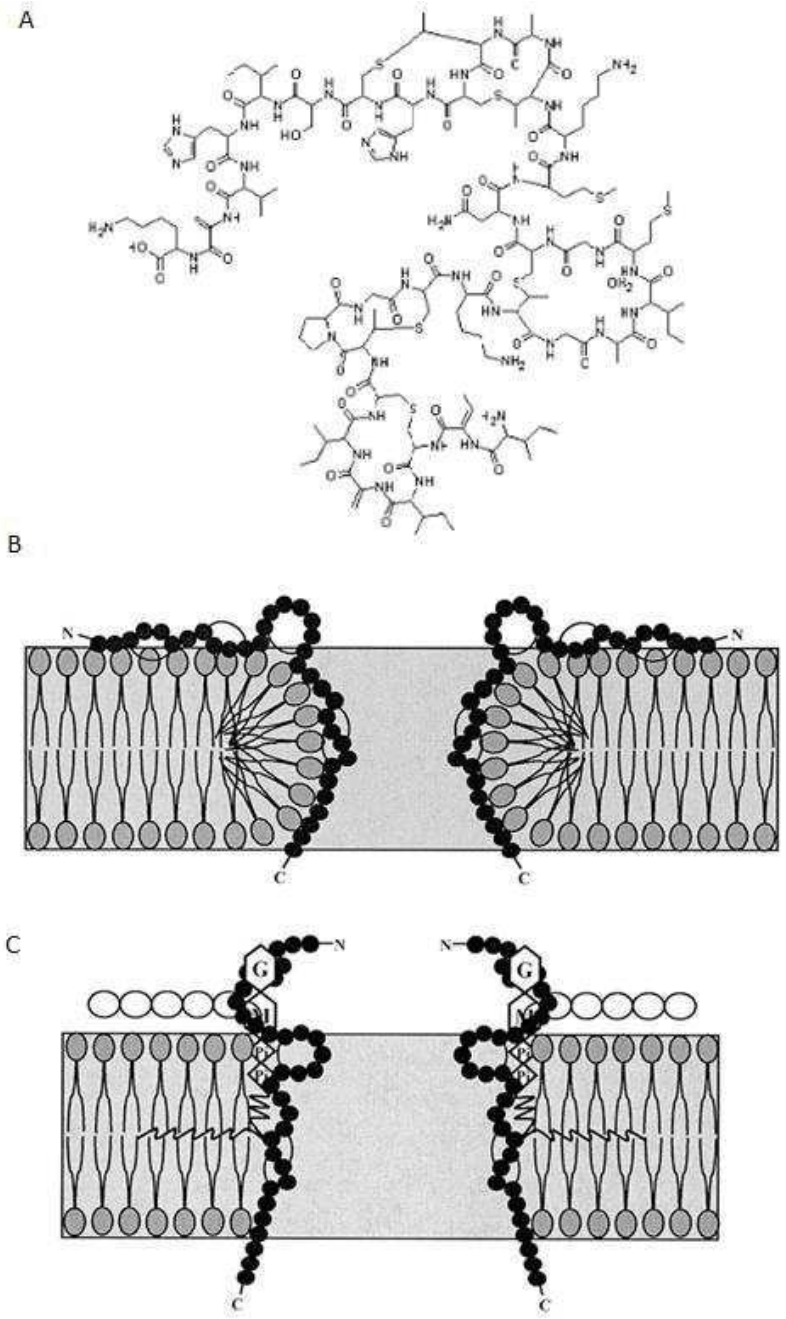


Figure 1.8. Structure and mode of action of nisin. (A) Structure of nisin [181]. (B) at micromolar concentrations, the cationic type-A peptides (nisin, epidermin, Pep5 and others) form wedge-like, target-independent pores. (C) at nanomolar concentrations, nisin and epidermin form target-mediated pores using lipid II as a docking molecule (figure taken from [99]).

The bacteriocin vancomycin has been successfully used in the past where standard antibiotics began to fail. The main concern for its use being its toxicity to the kidneys, but at low concentrations it is tolerated by the human body [168, 214]. However, it became a victim of its own success as some strains of resistant bacteria began to appear [128]. Vancomycin principally binds to the muramyl pentapeptide component of bacterial peptidoglycan,

inhibiting transglycosylation and transpeptidation, thus the formation of the cell wall, as shown in Figure 1.6. [128, 168, 245].

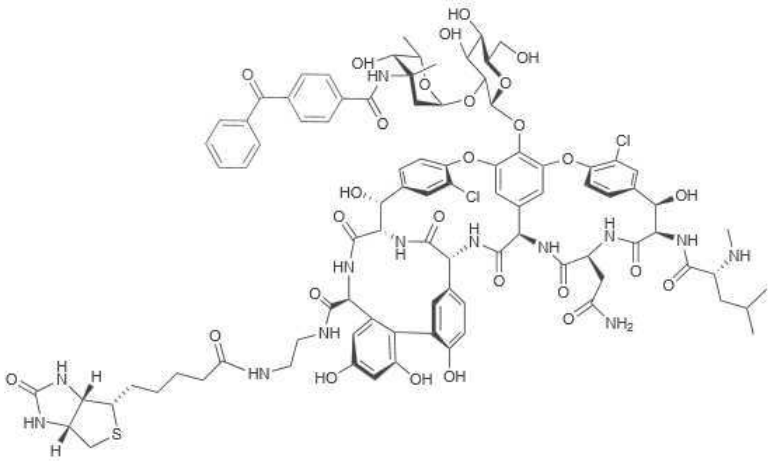


Figure 1.9. The structure of Vancomycin [128]

LL-37 is a human defensin of the cathelicidin family [23]. Mucosal surfaces, specifically neutrophils and epithelial cells express LL-37 in order to control the microbiota present in the gut, nasopharynx, skin etc.. LL-37 is in practice heavy artillery of the host innate immune system which can destroy biofilms, kill bacteria, fungi and viruses indiscriminately. All in all, LL-37 provides the first line of defense of human immunity as it may act on all microbial populations [170, 233, 239]. Consequently, it is absolutely essential to a healthy human life and protection from infections [23, 239]. LL-37 binds to the surface of cellular membrane, where it forms pores once it reaches the critical concentration, and the amphipathic domains penetrate the membrane [100, 113, 139, 217, 239].

Figure 1.10. 3D Structure of LL-37 resolved by NMR [243]. A superposition of five different structures to demonstrate the C-terminal disorder. The top of the helix is hydrophilic whereas the inside of the bend is hydrophobic.

Ramoplanin was discovered in 1984 and isolated from *Actinoplanes* sp. [172, 190]. It has been used in the past as an antibiotic against *Enterococci* which were resistant to vancomycin and other antibiotics [78, 95, 252]. Similarly to nisin, ramoplanin also binds to Lipid II in the bacterial membrane and inhibits cell wall formation and is capable of pore formation (Figure 1.6.). It is an extremely powerful compound effective at very low concentrations in the nM range [37, 78, 165].

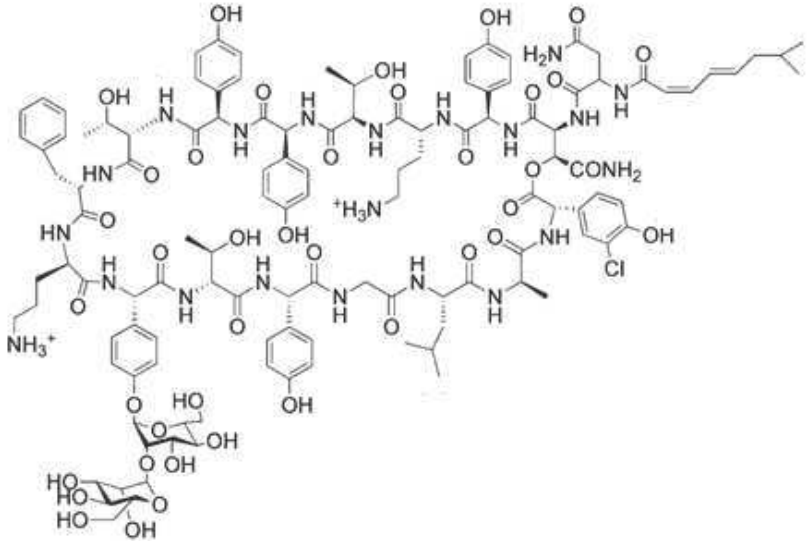


Figure 1.11. The structure of ramoplanin [95]

Duramycin is a lantibiotic, which has been discovered in 1958 and isolated from *Streptomyces cinnamomeus* [39, 191, 258]. It is active against only a few Gram-positive bacteria, especially in the genus *Bacillus*. However, it is also toxic to eukaryotic cells as it permeates cellular membrane by binding to phosphatidylethanolamine and diphosphatidylglycerol [19, 39, 99].

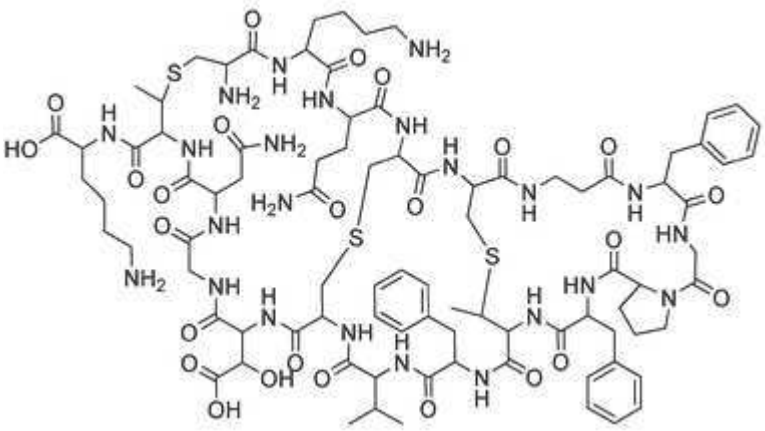


Figure 1.12. Structure of duramycin [53]

1.3. ATP-binding cassette (ABC) transporters

ABC proteins are a vast protein superfamily which is essential for the survival of all known cells; eukaryotes, prokaryotes and archae [36]. This superfamily is composed of transmembrane proteins involved in translocation of molecules as well as some non-transporting functions such as DNA/RNA repair. In humans, they were found to be involved also in membrane elasticity and lipid. Many genetic diseases and disorders, such as cystic fibrosis, can be traced back to an ABC transporter mutation [62, 213, 231]. There are a number of different ABC transporter classes depending on their sequence, structure and function. ABC transporters use the energy from ATP hydrolysis to transport molecules against the concentration gradient. ABC transporters are known to transport a number of various molecules, which range from nutrients, antibiotics, antimicrobial peptides, toxins, metals, ions, hormones etc.

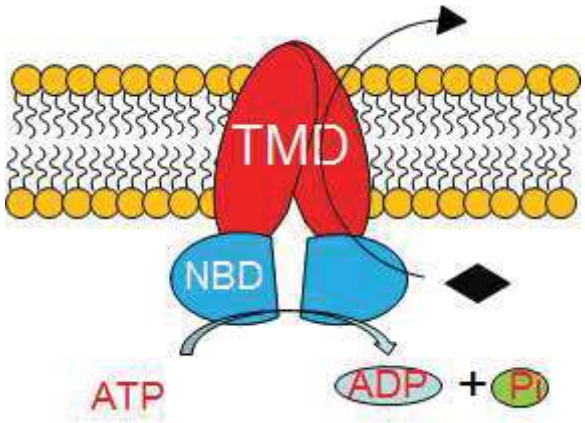


Figure 1.13. Typical ABC transporter. A dimer of transmembrane domains (TMD) and a dimer of nucleotide-binding domains (NBD) form together a complete ABC transporter, in this example an exporter.

1.3.1. ABC superfamily

ABC transporters have been discovered in the 1970s and studied in different prokaryotes. In the 1980's it has been discovered that a similar ATP-hydrolyzing protein was involved in a drug-resistant type of cancer [11, 29]. Today, thousands of different ABC transporters have been identified and classified into several families and in dozens of clusters. By sequence analysis, it has been shown many of these families are not kingdom specific. The

presence of these transporters in practically all living organisms suggests that it is a very ancient and common engine [49, 50].

In bacteria, ABC transporters pump a number of different compounds across the cellular membrane in either direction. For example, they transport lipids, cations, sugars, oligopeptides, amino-acids to mention a few [35, 101]. With such a wide range of substrates and a given cell expresses many different ABC transporters in order to sustain its viability. The ABC transporters are essential to bacterial life as they are responsible for nutrient uptake, such as iron, which is also an important virulence factor; they can correct osmotic pressure by either solute influx or efflux; they efflux toxic waste produced by the cell; they are involved in drug resistance; they are sometimes essential for pathogenicity; ABC type proteins also sometimes have housekeeping functions such as mRNA repair. ABC transporters represent up to 5% of the genome of many bacterial species (i.e. *E. coli, B. subtilis* [144, 254]); consequently it is clear that they are crucial for bacterial survival and their interaction with the environment [81].

ABC transporters are also responsible for resistance towards many drugs in a great variety of bacterial organisms. While there are some substrate-specific ABC transporters, many of them can transport a relatively wide variety of drugs. This phenotype is called multidrug resistance (MDR) and presents a veritable challenge to today's medicine. Many different proteins may produce MDR phenotypes in bacteria, such as MATE, MFS, small multidrug resistance (SMR) or resistance nodulation division (RND). ABC transporters are responsible for some multidrug resistances as well. The first known drug transporter is the human P-glycoprotein in cancer cells, which resisted cancer drugs [70, 237]. Its discovery helped to understand drug resistance in prokaryotes as well [10]. There are several ways in which the ABC transporters protect the bacteria from antibiotics. A common one is sensing and pumping the molecule directly from the membrane or cytoplasm out of the cell. They have most likely evolved from transporters effluxing drugs synthesized by the bacteria itself. A common substrate to the majority of ABC MDRs is ethidium bromide, which serves as a good reference substrate [32, 43, 141, 146, 244].

Because MDRs may potentially transport essential molecules as well as drugs, the systems must be regulated. For example, in humans the MRP2 multidrug transporter is tightly regulated on several levels such as transcription, translation and membrane reinsetion. When there is an overexpression without a drug present, it may lead to serious health problems such as epilepsy [85]. A similar system is in bacteria, where it is often the drug itself which is a positive regulator of the expression of the transporter [194, 234]. It has been described already

twenty years ago how low concentrations of a drug relieve the repression expression of the drug efflux pump in order to counter the toxicity [127, 203]. Some of these regulatory systems are TCS, which assure the resistance to drugs as well as down-regulation of efflux pumps when not necessary [89, 121].

1.3.2. Structure and mechanism

Despite the fact that ABC transporters are involved in many functions and transport an impressive variety of substrates in one direction or another, their overall topology stays highly similar [51]. Gram negative bacteria have ABC transporters only in the inner membrane. Gram positive bacteria, on the other hand, lack the outer membrane. All of the ABC transporters are in direct contact with the cytoplasm and the external milieu without the need to pass the ligand through the periplasm [51, 174].

1.3.2.1. General topology

Most commonly, ABC transporters are composed of two transmembrane domains (TMD) and two nucleotide binding domains (NBD). A single ABC transporter may be encoded for by two to five different genes [65]. The NBD domains (sometimes called ATP binding domains) are the engine burning ATP that, as a consequence, changes the conformation of the TMD and thus drives molecule transportation [51, 72]. The basic arrangement of ATP-bound NBDs is a dimer. It has been reported in several cases that NBDs can be encoded by two different genes, or that the NBD domain is already fused to the TMD in the genome. Therefore, regardless of the genetic organization or function of the ABC transporter, the basic tetrameric organization stays conserved (Figure1.14.) [102, 144].

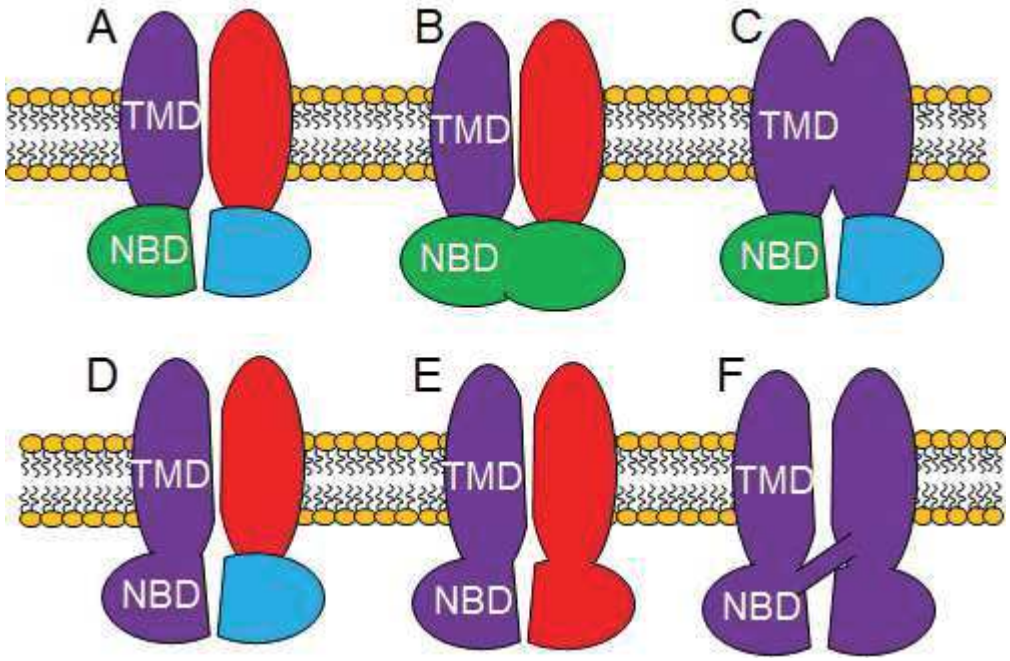


Figure 1.14. Schematic model of the organization of ABC transporters. The organization of the typical four domains of ABC transporters varies as follows. A) Four separate peptides e.g. OppBCDF (*E. coli*). B) NBD fused together e.g. ribose transporter RbsA (*B. subtilis*). C) TMD fused together e.g. FhuCB (*E. coli*). D) One TMD fused to one NBD and a complex of TMD and NBD e.g. YhiGHI (*E. coli*). E) Dimer of TMD and NBD fused together e.g. homodimers Sav1866 (*E. coli*), BmrA (*B. subtilis*) and heterodimer PatA/PatB (*S. pneumoniae*). F) All four domains fused together e.g. human ABCB1 [102, 144].

1.3.2.2. General description of transmembrane domains (TMD)

The transmembrane domain (TMD) is ordinarily composed of six transmembrane α-helices. One TMD then binds with another TMD to have a total of twelve transmembrane helices. These structures are highly variable depending on the substrate and the functioning mechanism of the protein. Consequently, the total number of the membrane spanning helices may vary between eight and twenty [51, 143]. Notwithstanding this variation, a weakly conserved common motif called the EAA loop (EAAXXXGXXXXXXXXXIXLP) may be found. This motif is predominantly present in importers, but may be found in some exporters as well [248]. Another conserved feature of TMDs is an FtsX-domain. Normally, four FtsX helices make up a transport chamber and regulate cell wall hydrolases [17].

ABC importers sometimes have a substrate-binding protein (SBP) associated to the permease or co-operating with the extracellular part of TMD. These SBPs are responsible for binding the substrate which is eventually transported by the protein. SBPs are in control of the importer specificity [14, 248]. The ligand specificity of ABC exporters is usually assured by the TMDs themselves.

1.3.2.3. General description of nucleotide-binding domains (NBD)

NBDs on the other hand are composed of several conserved motifs across ABC transporter classes regardless of the function (Figure 1.15.). These highly conserved motifs are not invariable though. They possess:

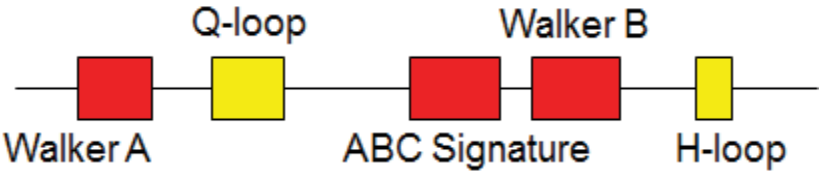


Figure 1.15. General organization of an NBD subdomain. In red Walker A, Walker B and ABC signature motifs are involved in binding and hydrolyzing ATP. In yellow Q-loop and H-loop have a single conserved residue and are also involved in hydrolyzing ATP as well as changing NBD/TMD conformation [51].

(i) Walker A motif involved in the binding of ATP: Walker A motif (or P-loop) is a glycine rich sequence surrounded on one side by a β-strand and an α-helix on the other side. The typical motif is GXXXXGKT/S where the lysine is crucial for ATP binding and hydrolysis. Upon ATP hydrolysis, the phosphate stays bound to the loop. Walker A is always found 100-190 residues upstream of the Walker B motif [195].

(ii) Walker B involved in hydrolyzing ATP: This motif has the following pattern (R)KXXXXGXXXXLhhhhD (h is a hydrophobic amino-acid) as defined by Walker in 1982. Today, it is accepted that only the hhhhD part is sufficient for Walker B recognition [51]. It is admitted that a conserved glutamate adjacent to the Walker B motif (hhhDE) together with the last acidic amino-acid polarize the attacking water molecule to hydrolyze ATP [177]. The aspartate of the Walker B motif extends to the active site and coordinated Mg^{2+} via a bound water molecule [195].

(iii) ABC signature sequence LSGGQ responsible for phosphate binding and locking the ATP to the Walker A motif of the other NBD monomer. As a result, it is necessary for dimer formation. The LSGGQ sequence is the beginning of a sequence of up to 15 residues long [208]. It is a conserved sequence present in all ABC transporters.

(iv) Q-loop is normally found between the Walker A motif and the ABC signature. It begins by a highly conserved glutamine and followed by several residues. It often interacts with the EAA loop of the TMD, generally forming a salt bridge. The Q-loop is involved in changing the TMD conformation [56, 248].

(v) H-loop is typically found downstream of the Walker B motif. It is a sequence that surrounds a histidine within the "switch" region involved in changing TMD conformation [51, 56].

Despite the difference in sequences of NBDs, the folding stays highly similar for all NBD domains suggesting a similar functioning mechanism [51]. To bind ATP, the NBDs form a dimer binding head-to-tail by the P-loop, Walker B, H-loop and the Q-loop of one NBD to the ABC signature motif of the other NBD (Figure 1.16.B) and locking the ATP molecule between them. Each dimer therefore has two ATP binding sites. Normally, NBDs form a dimer only when they are in an ATP-bound form. The mechanism of releasing ADP and the phosphate is still unknown. It is thought that upon ATP hydrolysis the NBDs dissociate and upon dissociation, ADP and phosphate detach themselves from the NBDs. Hydrolysis in a single site is sufficient for NBD dimer dissociation [14, 48, 51, 143, 198].
Surprisingly, only a few residues of the NBD interact with the base moiety of ATP, which might explain the fact that NBD binds other substrates as well, such as GTP. The lysine residue of the Walker A motif is essential for ATP binding. It forms hydrogen bonds with oxygen atoms of β and γ phosphates. The ATP is therefore locked into position and Mg^{2+} ion is anchored to the site by Walker A motif T or S residues. The LSGGQ motif is also required to correctly coordinate the γ-phosphate. The histidine from the H-loop then forms a hydrogen bond with the γ-phosphate in order to hydrolyze the ATP molecule [51, 195, 248].

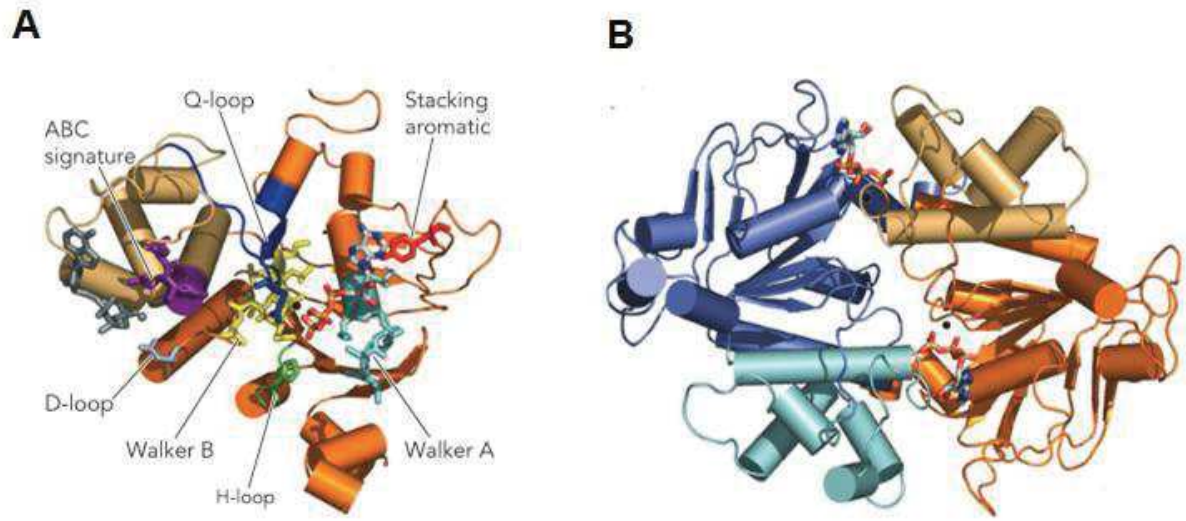


Figure 1.16. Model of *Staphylococcus aureus* Sav1866 drug exporter NBD structure. A) NBD monomer of Sav1866 showing conserved motifs. ATP binds to the Walker A and B and the H-loop. ABC signature motif extends itself to another ATP. Q-loop reaches out to the TMD. B) A dimer of NBDs. ATP molecules are between the Walker A of blue and ABC signature motif of golden subunit (and vice versa) (figure adapted from [145]).

1.3.2.4. Model ABC transporter: Sav1866

S. aureus drug transporter Sav1866 (homologous to LmrA from *L. lactis* and BmrA of *B. subtilis*) may serve as an adequate model of overall ABC mechanism [55]. The TMDs are between 70 and 80 Å long. The NBD is then found 25 Å away from the membrane in the cytoplasm (Figure 1.17.A) [10, 51]. The model proposed is called "the alternating model" and should be generally true for both importer and exporters. This complex forms two drug-binding sites and two ATP-binding sites. The TMDs form a high-affinity binding site towards the cytoplasm and a low-affinity site towards the external medium (Figure 1.17.B).

To export the drug, the TMDs first bind the drug in the cytoplasm where TM1-3 and 6 of one TMD bind to TM4 and 5 of the other. Then, the protein changes conformation so that it flips and presents the drug to the exterior with the low-affinity site. For the outward facing conformation the TM 1 and 2 are bound to TMs 3-6 of the other and forms two "wings" of TMDs (Figure 1.17.A) [51, 174, 175]. The cavity presented to the external milieu has relatively low hydrophobicity, which could explain the low affinity.

The TMD conformation change is driven by NBD hydrolyzing ATP. The NBD binds to the TMD through the Q-loop and the sequence between the Q-loop and the signature motif which is conformationally highly variable and two intracellular helices from the TMD [10, 174, 198]. Once the phosphate is cleaved off, the glutamine residue from the Q-loop and histidine residue, of the H-loop, gather at the site. By hydrolyzing the ATP, the Q-loop changes the

NBD conformation and drives conformational change of the TMD [10, 51, 143, 146, 198, 210].

Each NBD subunit interacts with its own TMD as well as the other TMD through the Q-loop and the TEVGERV motif (found only in exporters) before the ABC signature [51, 116, 174, 198]. It is important to note that upon the NBDs dissociation, the ADP and P_i are released in order to present the substrate binding site between the TMDs to the cytoplasm again [175].

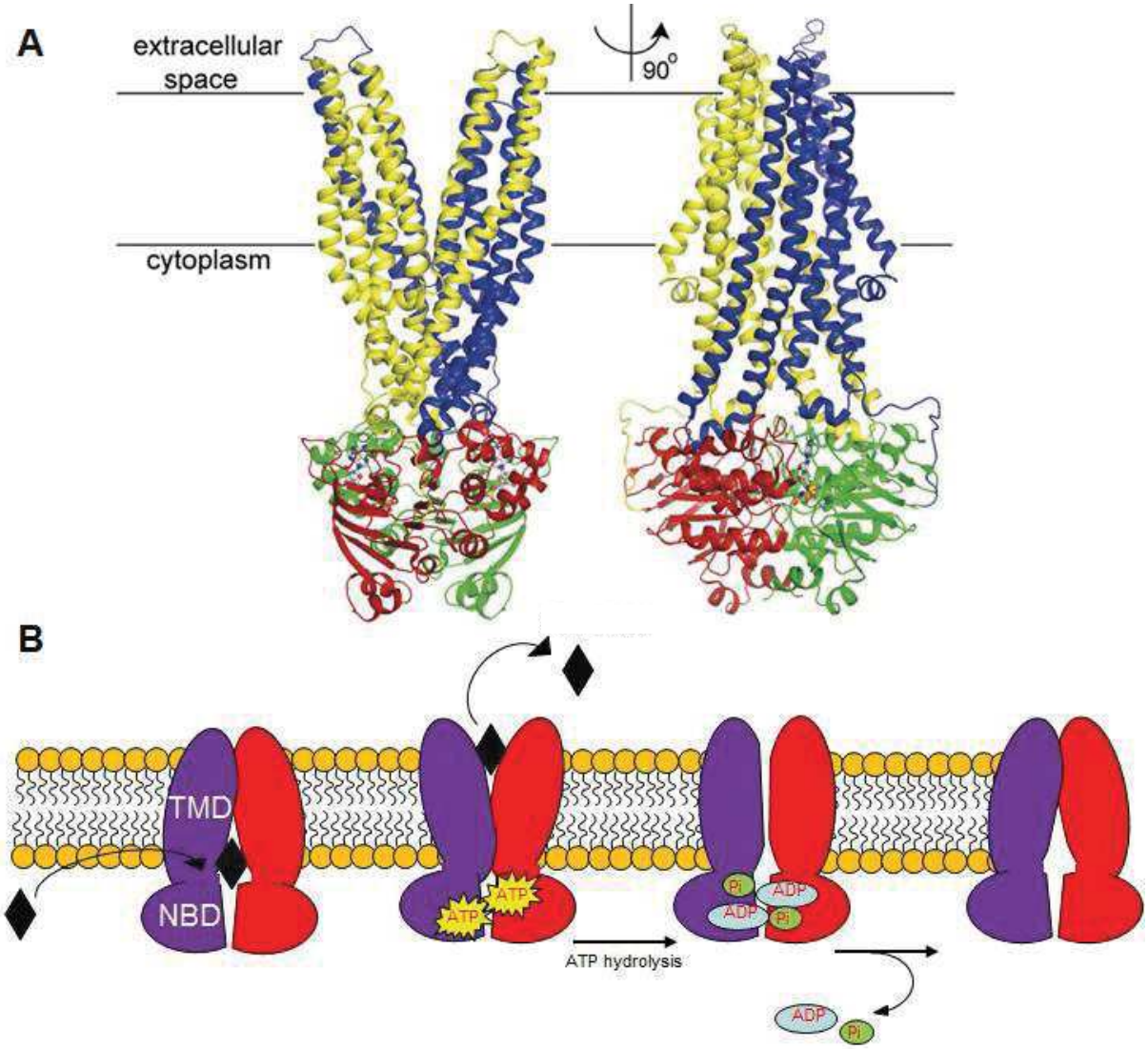


Figure 1.17. Structure of complete Sav1866 transporter and its mechanism model. A) ATP-bound Sav1866 transporter in the outwards facing conformation. One subunit blue (TMD) and green (NBD) is bound to the other yellow (TMD) and red (NBD). ADP and P_i are represented in ball and stick format (figure taken from [174]). B) Cartoon showing in the first step binding of a ligand to the TMD in the high affinity conformation towards the cytoplasm. Second step shows binding of ATP and release of the ligand. Towards step three, ATP is hydrolyzed. By releasing ADP and phosphate between steps three and four the ABC transporter returns to its original conformation [145].

ABC transporters require ATP to function, however the stoichiometry of ATP used per molecule transported is still not known for the majority of transporters. There have been several *in vitro* studies attempting to determine the ratio of ATP per substrate, but there is a vast variation between transporters studied [51-53, 70, 198]. It is generally accepted that small, simple substrates require less ATP to transport than larger ones, such as peptides [70]. For an exporter, to function as a pump, it must efflux a higher amount of substrate than comes into the cell. At the same time it is desirable to spend the minimum amount of ATP. A combination of different elements may be in place to maximize the effectiveness of the reaction: as for Sav1866, high-affinity site facing the cytoplasm and low-affinity site facing the exterior; binding ATP and hydrolysis only after the substrate binds to the protein (not true for all transporters); and quick ADP release to a 'ready' state [20, 24, 52, 70, 178, 198].

1.3.2.5. AMP transporters

AMPs have been presented previously in the chapter. Most of these compounds are targeted against Gram-positive bacteria. In the past, ABC transporters targeting these AMPs were considered as proteins functioning only for self-immunity or exporting synthesized peptides. Today, they are getting more attention, because it has been discovered they provide a wide arsenal of defense for Gram positive bacteria. There are several groups of these transporters with different modes of action, different substrate preference and different regulation. Many of them are regulated on one level or another by a TCS system, but the regulatory mechanism is quite variable as well [60, 200, 218]. In 2012 a review has been published proposing a classification system of these ABC transporters based on the predicted structures of the TMDs [83]. The author created five groups named after the most studied and characterized example of each group described below (Figure 1.18.):

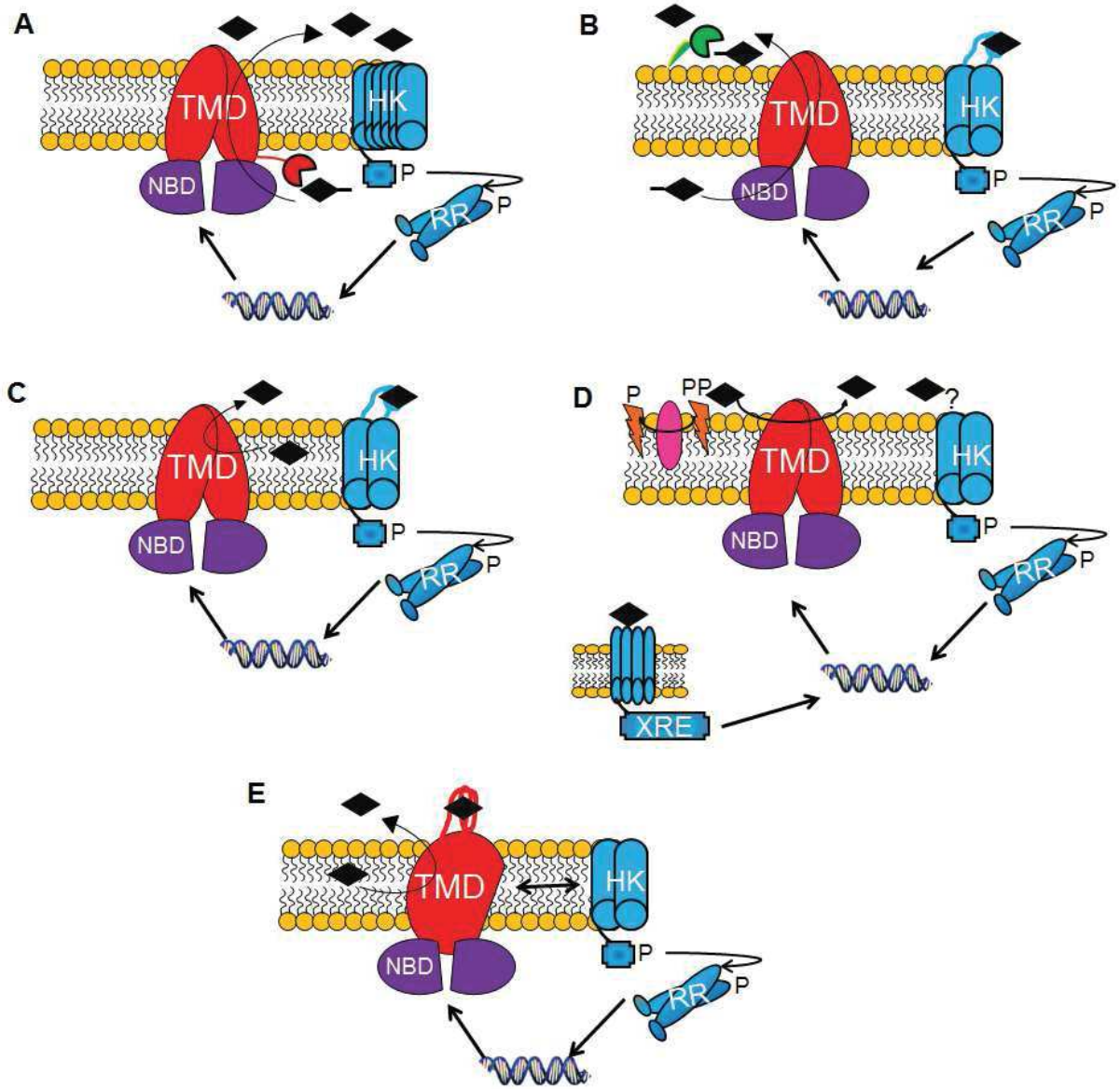


Figure 1.18. Five AMP transporter groups. Black rhombus represents an AMP. TMDs and its parts are in red. NBDs are represented in violet. Blue objects are regulators. "Pacman" represents a peptidase domain. Phosphorylated or unphosphorylated UPPs are shown as bolts. A) SunT type, with a peptidase domain on the *N*-terminus of TMD. B) NisT type exporter. Exported AMP is processed extracellularly. C) LanFEG type. The TCS serves as the sensor for the transporter. D) BcrAB has a complex regulation pathway. Main target is bacitracin. E) BceAB type transporter senses the AMP with an extracellular loop. It is regulated by a TCS [83].

- SunT-type is characterized by a peptidase domain on the N-terminus. SunT is responsible for transporting in-house produced AMPs and other peptides and likely controlled by a kinase system. It processes the AMP by the N-terminal peptidase domain before releasing the drug out of the cell [109, 169].
- NisT-type is an exporter of in-cell synthesized AMPs as well as foreign AMPs. Unlike SunT, NisT only translocates the AMPs which mature extracellularly. For some

representatives of this group a TCS system is present as a regulator as shown in Figure1.18.B [41, 236].

- LanFEG group has a very narrow range of substrates, either restricted to self-produced AMPs or very close relatives. The ABC transporter transports AMPs from the membrane into the extracellular media. LanFEG group is closely regulated by a TCS, which works as the AMP sensor [63, 193].
- BcrAB is the less frequent group with fewest representatives. It confers resistance mainly to bacitracin. It is the least known ABC transporter with a complex regulation pathway including a TCS system [71].
- The last but not least group is the BceAB type transporters, the focus of this PhD project, which are described in this introduction section 1.5.

Recently, a first crystal structure of a full AMP transporter has been obtained. This McjD transporter from *E. coli* exports MccJ25 AMP during starvation. The structure of this transporter with six α-helices is similar to those of Sav1866 and MsbA [36] and would fall into the SunT group proposed above (Figure 1.19.).

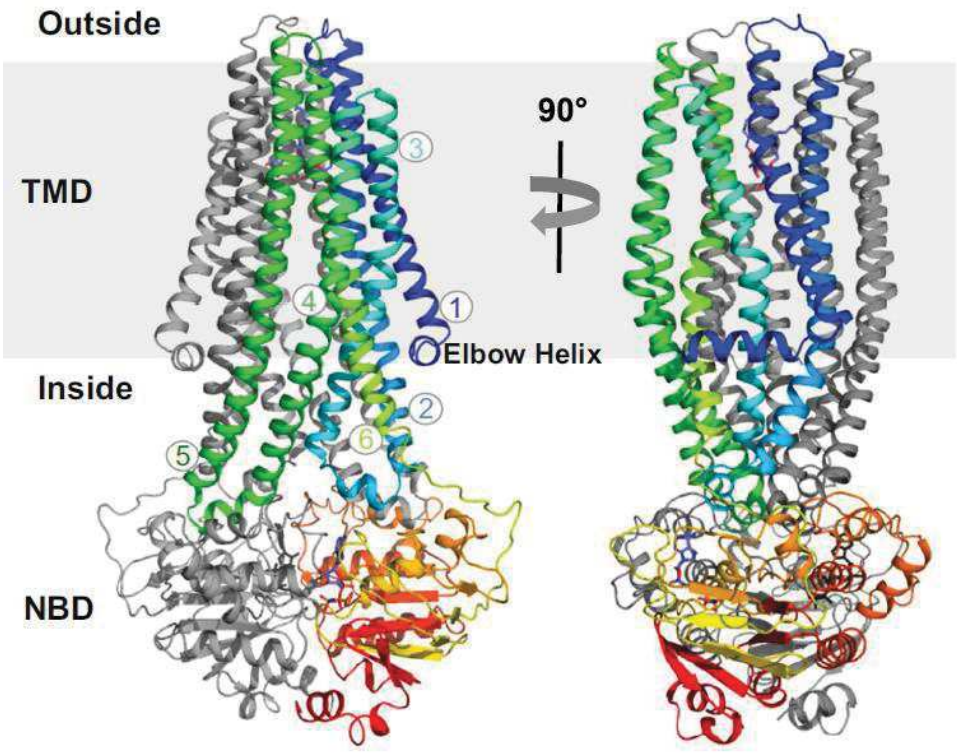


Figure 1.19. Crystal structure of *E. coli* McjD AMP transporter. The first crystal structure of an AMP transporter has been solved belonging to the SunT-type proteins (figure taken from [36]).

1.4. Two- component systems (TCS)

1.4.1. General description of TCS systems

The TCSs are found mainly in bacteria and archea and in a few eukarea. It is a basic stimulus and response mechanism. Throughout time it has adapted to respond to many different extra and even intra-cellular stimuli. They allow bacteria to regulate many essential functions, as nutrients uptake, chemotaxis, cell adhesion, sporulation like in *B. subtilis*, or antibiotic resistance for which *P. aeruginosa* is notoriously known [89]. However, the TCS system is not as universal as the ABC transporters. There are some bacteria with a substantial number of TCSs as *B. subtilis*, but there are other bacteria with none whatsoever as in *M. genitalium* [105, 137, 221]. Some of the major advantages of the TCSs include rapidity and specificity. In a few cases, HKs may able to cross-talk, or one HK might stimulate several RRs or several HKs are necessary to stimulate a single RR [137]. Some TCSs are seen as potential targets by small-molecule therapy where stimulating or shutting down a system might hinder bacterial viability [87].

1.4.2. Composition and mechanism

As stated above, the TCS is composed of at least two subunits: a membrane bound HK and a soluble RR. Even though HKs are a very diverse group, the typical HK is usually not longer than 400 amino-acids, has two transmembrane helices, an extracellular sensing loop and a conserved H-box, also called the dimerization histidine phosphotransfer domain (DHp) on the kinase domain [154, 221]. Once stimulated, the HK generally forms a homodimer and autophophorylates itself at the H-box from available ATP [46, 251]. The phosphate is then transferred to the aspartate residue of the RR. Many HKs also possess the ability to dephosphorylate the RR in order to quickly stop the response in the absence of stimulus [221]. In some cases, the binding of a substrate to the HK may inhibit basal autophosphorylation of the HK and therefore limit the signal transduction [221].

The RR is frequently substantially smaller than the HK, while its putative state is commonly a dimer. RR is composed of two domains, a receiver domain which includes a dimerization helix and a conserved Aspartate domain mentioned above and a diverse regulator domain, which is usually a DNA effector, but it may have other functions as well (Figure 1.20.) [46, 221]. The function and structure has been studied also thanks to several crystal structures of different RRs. Often, the RR can dephosphorylate itself to prevent an

overwhelming response, but there are a number of other mechanisms of dephosphorylation, such as auxiliary dephosphorylation proteins, some H-boxes of HKs, or other domains of the HK. Small molecules such as imidazole phosphate, or acetyl phosphate may cause autophosphorylation of the RR, but autodephosphorilation usually limits the active state duration [46, 57, 105, 158, 216, 221].

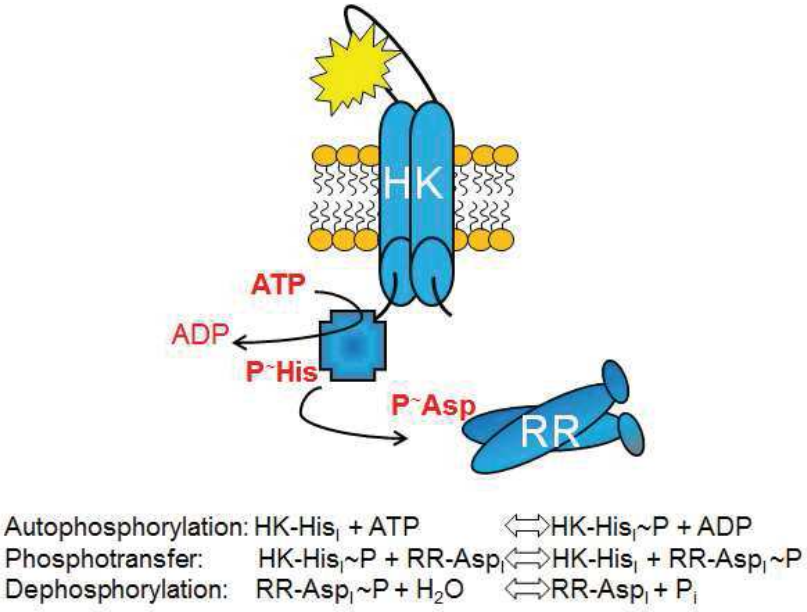


Figure 1.20. Model of a typical TCS and phosphorylation reactions: A typical histidine kinase (HK) senses a substrate, with an extracellular loop and through the H-box autophosphorylates itself from ATP. The H-box then transfers the phosphoryl group to the aspartate residue on the response regulator dimer (RR). The response regulator then performs its function and ultimately dephosphorylates.

1.4.3. Intramembrane sensing histidine kinases (HK)

Not all HKs, however, fit exactly into the typical scheme. There is a group of HKs, which have a very short extracellular loop (~5-25 amino-acids), which practically makes it impossible for them to be an extracellular sensor. On the cytoplasmic side, they have only the H-box, serving to phosphorylate the putative RR. Some of these HKs are called intra-membrane sensors. Out of the 5000 known HKs in prokaryotes, less than 200 fit into this category. Many of these proteins are found in Firmicutes bacteria, of which *S. pneumoniae* is a member. The majority of this type of HKs is linked to an ABC transporter involved in

detoxification of the bacteria and are an important element in cell envelop stress response [154-156].

1.5. ABC transporters and TCS

Sometimes, an ABC transporter has co-evolved with a two-component system. As we have shown above, TCS usually works as a regulator of ABC transporters as well as other processes. In the case of ABC transporters, the cognate TCS usually detects the presence of a stimulus and drive its transportation by the expression of a given transporter.

1.5.1. ABC and TCS in Pneumococcus

We have briefly introduced the Blp system above in section 1.1.3.4. Another example of ABC-TCS cooperation in pneumococcus is the competency described in section 1.1.3.1. A competence stimulating peptide (CSP) is transported out of the cell by the ABC transporter comAB. The comD HK then senses the peptide and activates the comE RR. The RR then has a number of targets, including upregulation of CSP expression and comAB expression. A total of 240 targets of the RR have been identified by microarray technology. Not all of them are regulated by the RR in a direct manner, but it activates other regulators that control gene expression (Figure 1.21.) [130, 153, 182].

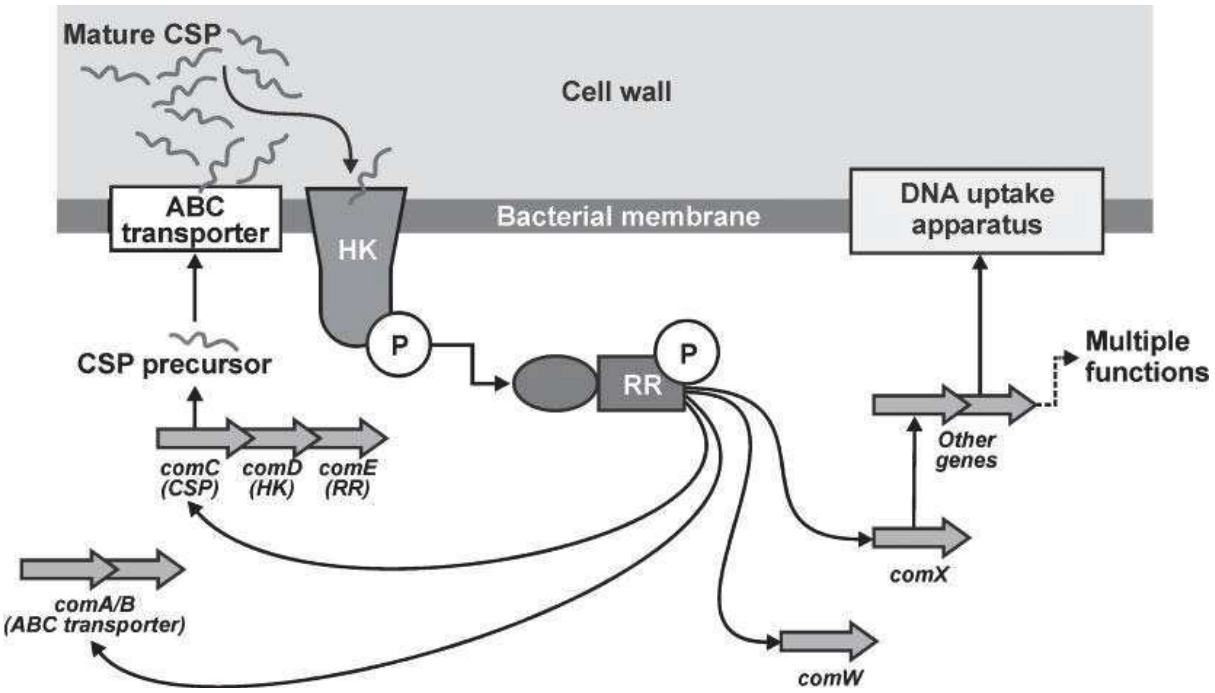


Figure 1.21. Competence regulation in *S. pneumoniae*. CSP is exported by the ABC transporter. The CSP then binds to the HK, which triggers a cascade, which upregulates CSP expression as well as the expression of the comAB ABC transporter. The RR regulates other genes and activates other regulators (figure adapted from [182]).

1.5.2. BceAB-RS type system

BceAB-RS complex belongs to the Peptide-7-Exporter family in the Transport Classification Database [207]. Although the BceAB-RS, formerly known as YtsAB-CD, system has been studied for more than a decade, progress in understanding of its functioning mechanism at the molecular level has been slow and modest. The first time an AMP resistance system similar to the BceAB-RS was described in *Salmonella typhimurium* in 1996 [93]. Afterwards, in early 2000's, the AMP resistance systems began to be studied especially in *B. subtilis*. Homologues of the BceAB-RS system have been found, among others, in *B. subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Lactobacillus casei*, *Enterococcus faecalis* and *S. pneumonia* [83].

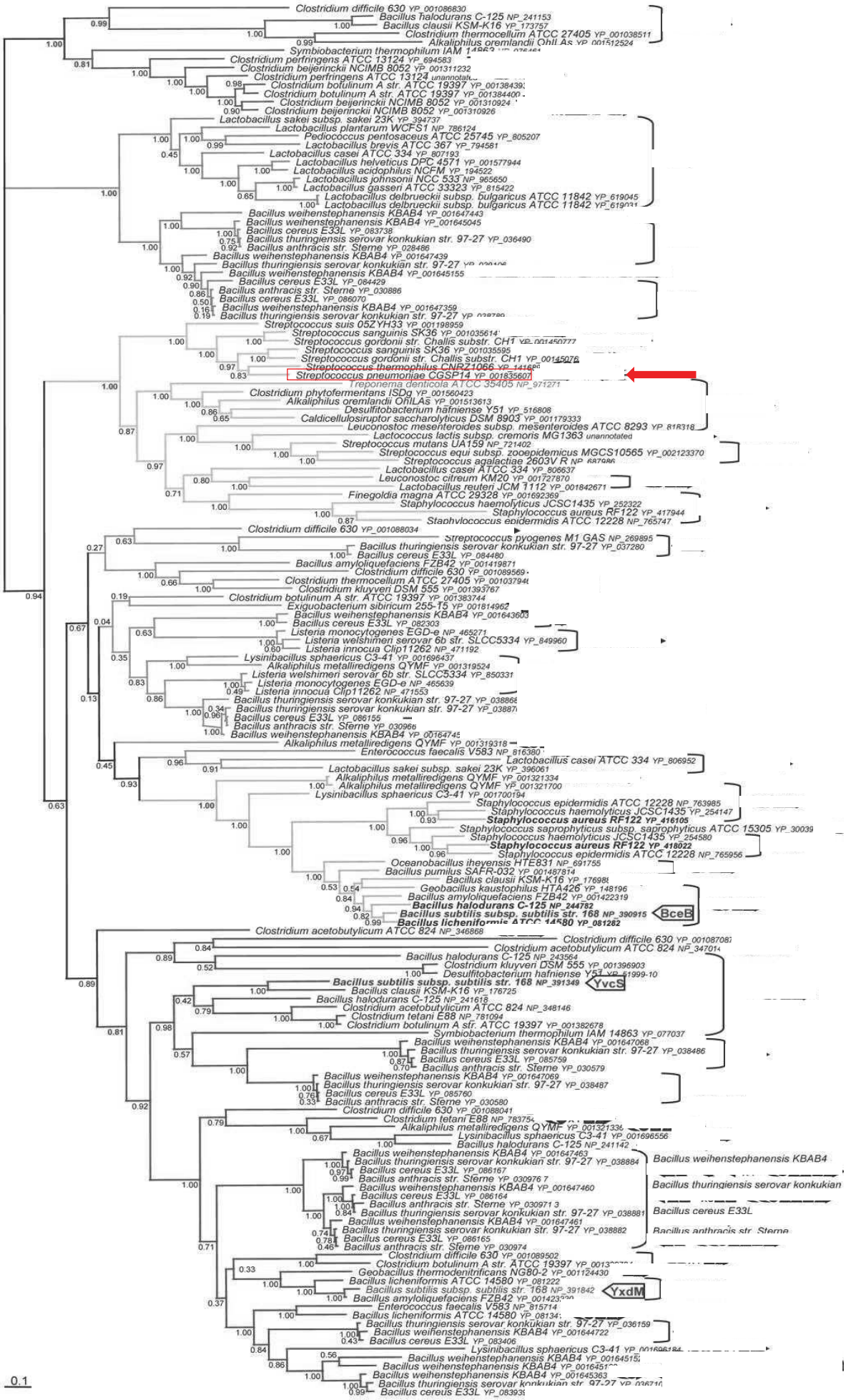

Clostridium difficile 630 YP_001086830
Bacillus halodurans C-125 NP_241153
Bacillus clausii KSM-K16 YP_173757
Clostridium thermocellum ATCC 27405 YP_001038511
Alkaliphilus oremlandii OhILAs YP_001512524
Symbiobacterium thermophilum IAM 14863 YP_076461
Clostridium perfringens ATCC 13124 YP_694583
Clostridium beijerinckii NCIMB 8052 YP_001311232
Clostridium perfringens ATCC 13124 unannotated
Clostridium botulinum A str. ATCC 19397 YP_001138439
Clostridium botulinum A str. ATCC 19397 YP_001384400
Clostridium beijerinckii NCIMB 8052 YP_001310924
Clostridium beijerinckii NCIMB 8052 YP_001310926
Lactobacillus sakei subsp. sakei 23K YP_394737
Lactobacillus plantarum WCFS1 NP_786124
Pediococcus pentosaceus ATCC 25745 YP_805207
Lactobacillus brevis ATCC 367 YP_794581
Lactobacillus casei ATCC 334 YP_807193
Lactobacillus helveticus DPC 4571 YP_001577944
Lactobacillus acidophilus NCFM YP_194522
Lactobacillus johnsonii NCC 533 NP_965650
Lactobacillus gasseri ATCC 33323 YP_815422
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 YP_619045
Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 YP_619031
Bacillus weihenstephanensis KBAB4 YP_001647443
Bacillus weihenstephanensis KBAB4 YP_001645045
Bacillus cereus E33L YP_083738
Bacillus thuringiensis serovar konkukian str. 97-27 YP_036490
Bacillus anthracis str. Sterne YP_028486
Bacillus weihenstephanensis KBAB4 YP_001647439
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038406
Bacillus weihenstephanensis KBAB4 YP_001645155
Bacillus cereus E33L YP_084429
Bacillus anthracis str. Sterne YP_030886
Bacillus cereus E33L YP_086070
Bacillus weihenstephanensis KBAB4 YP_001647359
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038780
Streptococcus suis 05ZYH33 YP_001198959
Streptococcus sanguinis SK36 YP_001035614
Streptococcus gordonii str. Challis substr. CH1 YP_001450777
Streptococcus sanguinis SK36 YP_001035595
Streptococcus gordonii str. Challis substr. CH1 YP_001450076
Streptococcus thermophilus CNRZ1066 YP_143456
Streptococcus pneumoniae CGSP14 YP_001835607
Treponema denticola ATCC 35405 NP_971271
Clostridium phytofermentans ISDg YP_001560423
Alkaliphilus oremlandii OhILAs YP_001513613
Desulfitobacterium hafniense Y51 YP_516808
Caldicellulosiruptor saccharolyticus DSM 8903 YP_001179333
Leuconostoc mesenteroides subsp. mesenteroides ATCC 8293 YP_818318
Lactococcus lactis subsp. cremoris MG1363 unannotated
Streptococcus mutans UA159 NP_721402
Streptococcus equi subsp. zooepidemicus MGCS10565 YP_002123370
Streptococcus agalactiae 2603V R NP_687986
Lactobacillus casei ATCC 334 YP_806637
Leuconostoc citreum KM20 YP_001727870
Lactobacillus reuteri JCM 1112 YP_001842671
Finegoldia magna ATCC 29328 YP_001692369
Staphylococcus haemolyticus JCSC1435 YP_252322
Staphylococcus aureus RF122 YP_417944
Staphylococcus epidermidis ATCC 12228 NP_765747
Clostridium difficile 630 YP_001088034
Streptococcus pyogenes M1 GAS NP_269895
Bacillus thuringiensis serovar konkukian str. 97-27 YP_037280
Bacillus cereus E33L YP_084480
Bacillus amyloliquefaciens FZB42 YP_001419871
Clostridium difficile 630 YP_001089569
Clostridium thermocellum ATCC 27405 YP_001037946
Clostridium kluyveri DSM 555 YP_001393767
Clostridium botulinum A str. ATCC 19397 YP_001383744
Exiguobacterium sibiricum 255-15 YP_001814962
Bacillus weihenstephanensis KBAB4 YP_001643603
Bacillus cereus E33L YP_082303
Listeria monocytogenes EGD-e NP_465271
Listeria welshimeri serovar 6b str. SLCC5334 YP_849960
Listeria innocua Clip11262 NP_471192
Lysinibacillus sphaericus C3-41 YP_001696437
Alkaliphilus metalliredigens QYMF YP_001319524
Listeria welshimeri serovar 6b str. SLCC5334 YP_850331
Listeria monocytogenes EGD-e NP_465639
Listeria innocua Clip11262 NP_471553
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038866
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038874
Bacillus cereus E33L YP_086155
Bacillus anthracis str. Sterne YP_030966
Bacillus weihenstephanensis KBAB4 YP_001647455
Alkaliphilus metalliredigens QYMF YP_001319318
Enterococcus faecalis V583 NP_816380
Lactobacillus casei ATCC 334 YP_806952
Lactobacillus sakei subsp. sakei 23K YP_396061
Alkaliphilus metalliredigens QYMF YP_001321334
Alkaliphilus metalliredigens QYMF YP_001321700
Lysinibacillus sphaericus C3-41 YP_001700194
Staphylococcus epidermidis ATCC 12228 NP_763985
Staphylococcus haemolyticus JCSC1435 YP_254147
Staphylococcus aureus RF122 YP_416105
Staphylococcus saprophyticus subsp. saprophyticus ATCC 15305 YP_30039
Staphylococcus haemolyticus JCSC1435 YP_254580
Staphylococcus aureus RF122 YP_418022
Staphylococcus epidermidis ATCC 12228 NP_765956
Oceanobacillus iheyensis HTE831 NP_691755
Bacillus pumilus SAFR-032 YP_001487814
Bacillus clausii KSM-K16 YP_176981
Geobacillus kaustophilus HTA426 YP_148196
Bacillus amyloliquefaciens FZB42 YP_001422319
Bacillus halodurans C-125 NP_244782
Bacillus subtilis subsp. subtilis str. 168 NP_390915
BceB
Bacillus licheniformis ATCC 14580 YP_081262
Clostridium acetobutylicum ATCC 824 NP_346868
Clostridium difficile 630 YP_001087081
Clostridium acetobutylicum ATCC 824 NP_347014
Bacillus halodurans C-125 NP_243564
Clostridium kluyveri DSM 555 YP_001396903
Desulfitobacterium hafniense Y51 YP_51999-10
Bacillus subtilis subsp. subtilis str. 168 NP_391349
YvcS
Bacillus clausii KSM-K16 YP_176725
Bacillus halodurans C-125 NP_241618
Clostridium acetobutylicum ATCC 824 NP_348146
Clostridium tetani E88 NP_781094
Clostridium botulinum A str. ATCC 19397 YP_001382678
Symbiobacterium thermophilum IAM 14863 YP_077037
Bacillus weihenstephanensis KBAB4 YP_001647068
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038486
Bacillus cereus E33L YP_085759
Bacillus anthracis str. Sterne YP_030579
Bacillus weihenstephanensis KBAB4 YP_001647069
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038487
Bacillus cereus E33L YP_085760
Bacillus anthracis str. Sterne YP_030580
Clostridium difficile 630 YP_001088041
Clostridium tetani E88 NP_783754
Alkaliphilus metalliredigens QYMF YP_001321336
Lysinibacillus sphaericus C3-41 YP_001696556
Bacillus halodurans C-125 NP_241142
Bacillus weihenstephanensis KBAB4 YP_001647463
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038884
Bacillus cereus E33L YP_086167
Bacillus anthracis str. Sterne YP_030976 7
Bacillus weihenstephanensis KBAB4 YP_001647460
Bacillus cereus E33L YP_086164
Bacillus anthracis str. Sterne YP_030971 3
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038881
Bacillus weihenstephanensis KBAB4 YP_001647461
Bacillus thuringiensis serovar konkukian str. 97-27 YP_038882
Bacillus cereus E33L YP_086165
Bacillus anthracis str. Sterne YP_030974
Bacillus weihenstephanensis KBAB4
Bacillus thuringiensis serovar konkukian
Bacillus cereus E33L
Bacillus anthracis str. Sterne
Clostridium difficile 630 YP_001089502
Clostridium botulinum A str. ATCC 19397 YP_001384744
Geobacillus thermodenitrificans NG80-2 YP_001124430
Bacillus licheniformis ATCC 14580 YP_081222
Bacillus subtilis subsp. subtilis str. 168 NP_391842
YxdM
Bacillus amyloliquefaciens FZB42 YP_001423730
Enterococcus faecalis V583 NP_815714
Bacillus licheniformis ATCC 14580 YP_08134
Bacillus thuringiensis serovar konkukian str. 97-27 YP_036159
Bacillus weihenstephanensis KBAB4 YP_001644722
Bacillus cereus E33L YP_083406
Lysinibacillus sphaericus C3-41 YP_001696184
Bacillus weihenstephanensis KBAB4 YP_001645153
Bacillus weihenstephanensis KBAB4 YP_001645147
Bacillus weihenstephanensis KBAB4 YP_001645363
Bacillus thuringiensis serovar konkukian str. 97-27 YP_036710
Bacillus cereus E33L YP_083939
0.1

Figure 1.22. Phylogenetic tree of 164 BceB-like proteins. The phylogenetic tree shows the versatility of the BceAB-RS like complex in Firmicutes bacteria. The scale bar indicates the average number of substitutions per site. Numbers at nodes show posterior probability. The red box and plain arrow point to the single BceB found in *S. pneumoniae*. Black arrows show the three BceAB-RS like complexes in *B. subtilis*. (figure adapted from [45])

Coumes-Florens *et al*. performed a phylogenetic study in 2011, blasting each of the four Bce components individually against the genomes of various bacteria. The results show that the system is practically unique to the Firmicutes bacteria with very few exceptions, therefore, it is possible that the system has originated in this phylum (Figure 1.22.). As with all ABC transporters and TCS there is significant variation between different species and strains. Some strains have several of these systems, some have a single one and others have orphan ABCs or TCSs of either one of the four components [45, 60]. The BceAB-RS family members can be phylogenetically split into six groups with a number of outliers unable to be categorized. This grouping does not seem to have any influence on the functioning mechanism though [60, 84, 200].

BceAB represents the ABC transporter part of the system and BceRS the TCS part. BceB subunit represents an atypical TMD. It has ten putative transmembrane helices with a large extracellular domain of about 200 amino-acids between transmembrane helices VII and VIII. This loop his highly variable and may have significant differences even between strains of the same bacterial species [83, 120, 201, 225]. Fascinatingly, it has been shown by Staron *et al.* in 2011 [218] that two AMPs of similar structure such as enduramycin and ramoplanin are not recognized by the same BceB subunit. BceA, the NBD, does not present any major variation in its primary sequence from a typical NBD as found in many other ABC transporters. BceS, the HK of the system, falls into the small category of IM-HKs. It has two transmembrane helices with a ~12 amino-acid extracellular loop, which presumably cannot bind any ligands [13, 45, 59]. The RR, BceR, is again a typical one.

The mechanism of the BceAB-RS complex is quite simple (Figure 1.23.). Because it has been described principally in *B. subtilis*, it is used as a universal model. The BceB binds bacitracin with the large extracellular loop and sends a message to the BceS, which autophosphorylates. The H-box then phosphorylates the aspartate of the BceR, which in turn binds to the promoter to express the BceAB [173]. BceA hydrolyzes ATP in order to transport the AMP [59, 60, 71, 83].

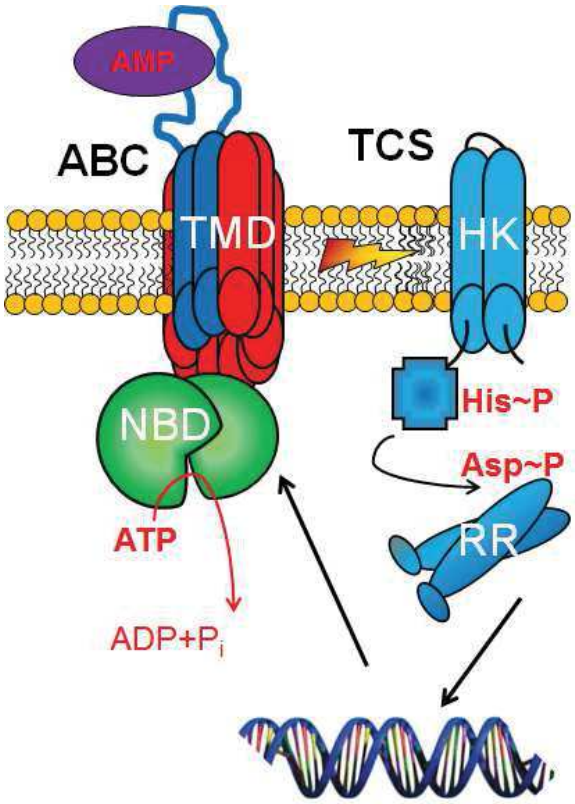


Figure 1.23. Schematic representation of the BceAB-RS system in the *B. subtilis*. The transmembrane domain (TMD) has ten predicted transmembrane helices, which is unusual for an ABC transporter. Helices 7 and 8 are very variable and especially the large extracellular loop of about 200 amino acids in length. This is the sensing loop which binds antimicrobial peptides (AMP), for example bacitracin. Once the AMP is bound to the loop, the BceAB transporter communicates with the histidine kinase (HK) of the BceRS TCS through an unknown mechanism. The HK then phosphorylates the aspartate of the response regulator (RR) through the H-box. The RR then upregulates the expression of the entire ABC transporter.

1.5.2.1. BceAB-RS type system in *B. subtilis*

The BceAB-RS like system has been studied most extensively in *B. subtilis* as it was chosen as a model for the complex. The genome of the bacterium contains different homologues with the same topology, namely PsdRS-AB (formerly YvcRS-PQ) and YxdLMJK, which are involved in nisin, vancomycin, LL-37 and other AMP transport [218]. BceAB-RS belongs to the phylogenetic group four whose main substrate is bacitracin, while the other two to group three, which may be triggered by LL-37, enduracidin, vancomycin etc. TCS and ABC genes are organized in the same operon, where the two TCS genes precede the two ABC genes in all three cases (Figure 1.25.). It has been clearly demonstrated that the BceAB-RS absolutely needs the complete BceAB type ABC transporter and BceRS type TCS in order to confer bacitracin resistance [13, 173]. The complex therefore needs all of the components to maintain its function [13, 199]. Even the integrity of the components is

important. For example, mutants lacking the large extracellular loop of the BceB were unable to trigger the system and lacked the resistance towards bacitracin as shown in 2007 by Bernard *et al.* [13]. Mutations in the BceA ATP binding motifs also increased bacitracin sensitivity. BceA ATP hydrolysis is consequently necessary for signaling [13, 83]. When the HK, BceS was deleted, no communication occurred with the BceR, which is essential for transcription of the BceAB. The molecular details of the signaling between the BceB and the BceS remains a mystery [120, 156, 225].

The exact stoichiometry of the BceAB and of the BceAB-RS complexes is still debated. Normally, one would expect the BceB to be a dimer as most TMDs of ABC transporters, however it has already ten predicted transmembrane helices and helices II-IV and VIII-X contain enough FtsX domains necessary to form a chamber for transportation [83]. A recent study by Dintner *et al.* [59], which is also the only one published on this subject, shows that while BceB is a monomer, BceA forms a dimer while in complex with the BceB. Moreover, their *in vitro* experiments suggest that BceAB and BceS form a complex. They coupled BceS to beads and washed them with purified BceAB. BceS and BceAB then co-purified during elution. The troubling aspect of this study is that the BceAB transporter did not show any ATPase activity.

Another unanswered question remains whether the ABC transporter is an importer or an exporter. While its predicted topology and bioinformatics analysis suggest that it is an exporter, no experimental data has been produced to support the claim [83], other studies suggest it is an importer [201]. Another recent study [125] suggests that it is not bacitracin itself, which is the substrate of the transporter, but UPP. Supposedly, the BceAB flips the bacitracin-bound UPP into the cytosol, which is the mechanism that triggers the BceRS response. The claim has to be confirmed *in vitro* as well as with other AMPs. Specifically BceAB in *B. subtilis* also transports mersacidin, plectasin and actagardine which also inhibit cell wall synthesis, but have a different mode of action than bacitracin. The homologues of BceAB-RS, PsdRS-AB and YxdLM-JK are triggered by AMPs with yet another mode of action, therefore UPP flipping cannot be a universal answer to the mechanism of the BceAB-RS like complexes.

1.5.2.2. BceAB-RS type system in *E. faecalis*

Similar systems were found in *E. faecalis*. In this case, there are however two different BceAB type ABC transporters working together with one BceRS type TCS. Unlike *B. subtilis* or other bacteria, the two BceAB transporters EF2050-49 and EF2752-51 do not have a

BceRS in the same operon, or in their close neighborhood [84]. The homologous BceRS-like TCS (EF0926-27) is in another operon with overlapping ORFs, quite distant from the two ABC operons (Figure 1.25.). The EF2050-49 transporter belongs to the group five, but the other could not be classified. The EF2752-51 has homologues in *E. faecalis* and several *Lactobacilli* [60, 83, 84].

Both of the ABC transporters are BceAB like transporters where, the BceB type TMD part has ten transmembrane helices with a large extracellular loop between the transmembrane helices VII and VIII. The loop senses bacitracin and EF2752-51 sends a message to the BceRS, which in turn overexpresses the two ABC transporters, but EF2050-49 at a higher rate than the other (Figure 1.24.). Both of the transporters are necessary for full bacitracin resistance. When *E. faecalis* EF2050-49 gene replaced the BceAB gene in *B. subtilis* genome, the *E. faecalis* protein replaced the function of *B. subtilis* protein. On the other hand EF2752-51 was not able to trigger transcription from the BceAB promoter [60, 84]. Nisin, gallidermin, vancomycin, teicoplanin, and penicillin G did not trigger a response, while mersacidin did [71, 83, 84].

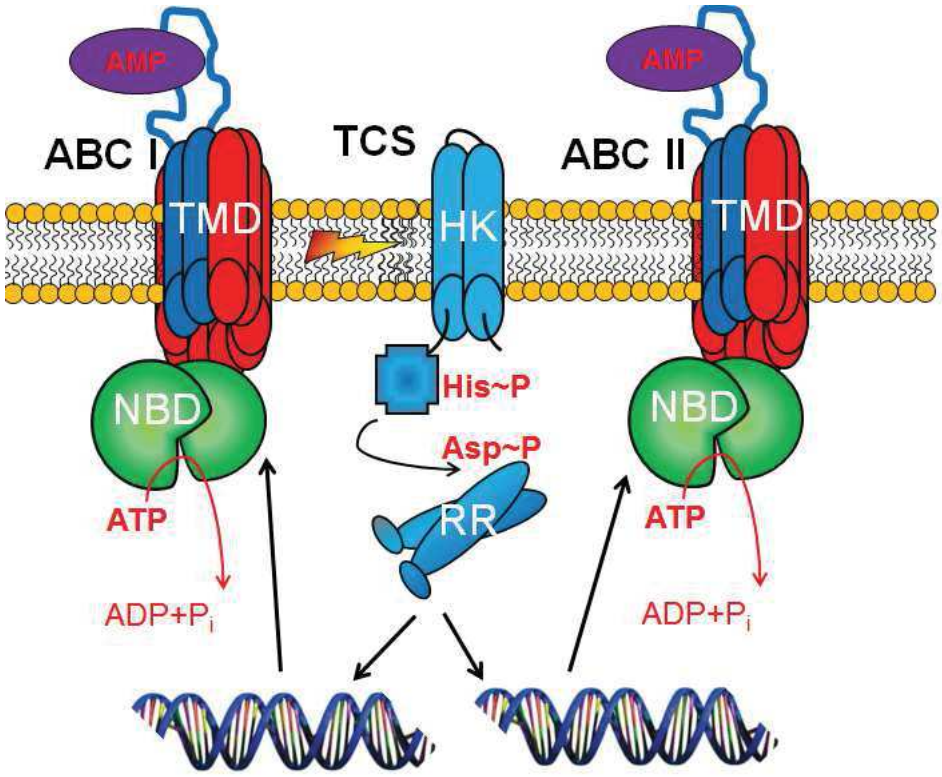


Figure 1.24. Schematic representation of the BceAB-RS like system in the *E. faecalis*. The transmembrane domain (TMD) have ten predicted transmembrane helices, which is unusual for an ABC transporter. Helices 7 and 8 are very variable and especially the large extracellular loop of about 200 amino acids in length. This appears to be the sensing loop which binds antimicrobial peptides (AMP), for example bacitracin. Once the AMP is bound to the loop, only one of the BceAB type ABC transporters (EF2752-51 on the left) sends a message to the histidine kinase (HK) of the BceRS type TCS through an unknown mechanism. It is suspected that helix 5 is responsible for this action. The HK then phosphorylates the aspartate of the response regulator (RR) through the H-box. The RR then upregulates the expression of both ABC transporters, but the EF2050-49 (ABC II) at a higher level than the sensor.

1.5.2.3. BceAB-RS type system in other organisms

Other organisms in which the BceAB-RS-like system was looked at in some detail include: *S. aureus*, *L. casei*, *S. thermophiles*, *S. mutans* and *L. monocytogenes* [83]. *S. aureus* and *L. casei* both have three BceAB-like transporters and two BceRS-like TCSs. While in *S. aureus* one of the TCSs works together with two ABCs [43, 121] and the other seems like a regular BceAB-RS complex, in *L. casei* there is one orphan Bce-AB-like ABC transporter which does not seem to be liked to a TCS and is likely to transport nisin on its own [180, 199, 228]. *S. thermophiles*, *S. mutans* and *L. monocytogenes* all have a single BceAB-RS like system. *S. mutans* has even the same operon organization as *B. subtilis*. The mechanism or description of the systems is consistent with those examples described above [43, 121, 180, 199, 228].

1.5.2.4. BceAB-RS type system in *S. pneumoniae*

To confirm the assumption form Figure 1.22., that there is only one BceAB-RS like complex in *S. pneumoniae*, we compared its genome to the genomes of *B. subtilis* and *E. faecalis* using the BLAST software. When any one of the four components of the multiple BceAB-RS systems form *B. subtilis* or *E. faecalis* was used as a query, a unique TMD/NBD couple and HK/RR couple were identified in the pneumococcus. The system is classified in group two according to the phylogenetic tree [45, 60]. The ORF organization is more similar to the one in *E. faecalis* than the one in *B. subtilis*. The BceAB type ABC transporter is in the same operon and the NBD (BceA) and the TMD (BceB) are separated by a single thymine, while the BceRS type TCS is in another operon with overlapping ORFs quite distant of the ABC transporter genes (Figure 1.25.).

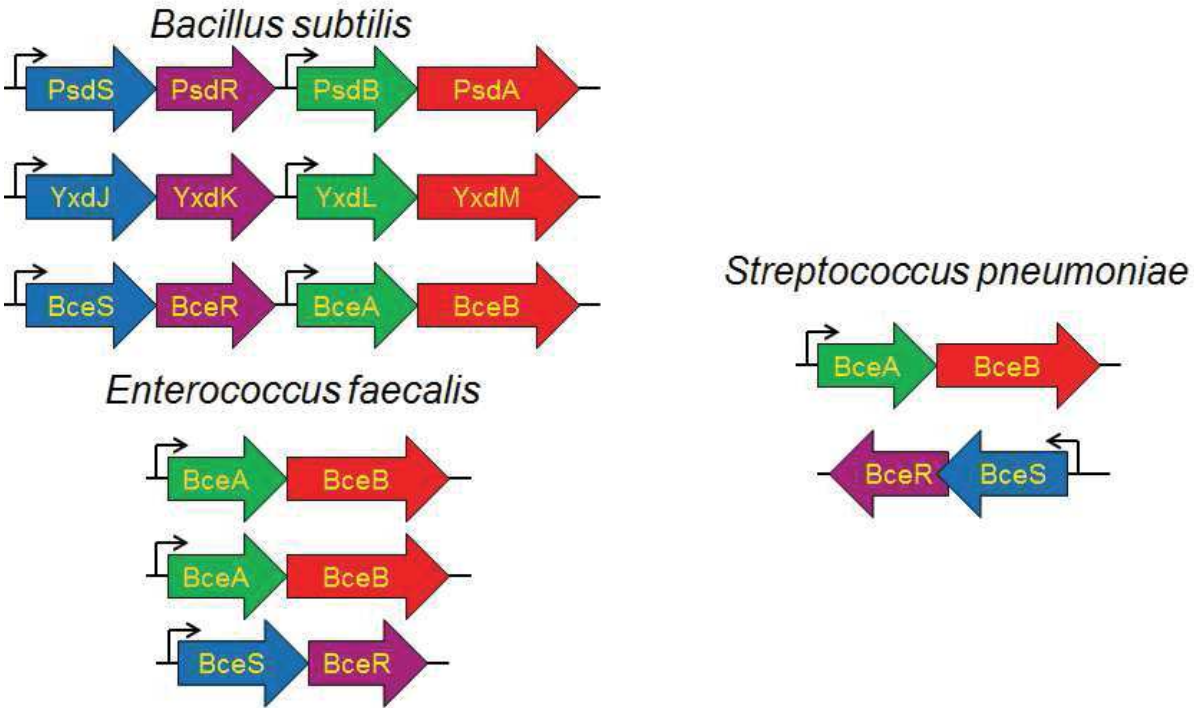


Figure 1.25. Schematic representation of the resistance operons, identified or putative, in *B. Subtilis*, *E. faecalis* and *S. pneumoniae D39*. Bent arrows represent gene promoters. Red represents the TMD, green represents NBD, blue represents HK and violet arrows represent RR. In *B. subtilis* there are three similar ABC transporters and TCS systems which provide resistance towards antimicrobial peptides. In *E. faecalis* there is one single TCS system which works together with two BceAB type transporters. When we run a BLAST with each of these genes against the *S. pneumoniae D39* genome a single ABC transporter and TCS are identified.

A study conducted by Becker et al. in 2009 demonstrated that in the non-pathogenic *S. pneumoniae* R6 strain, the mutation in BceB led to a higher sensitivity of the bacteria towards

bacitracin, however a truncated TMD reduced the susceptibility to vancoresmycin. Majchrzykiewicz et al. in 2010 showed the induction of expression of the BceAB in D39 strain of the *S. pneumoniae* by bacitracin and nisin, but not by vancomycin [12, 150]. A higher expression level of BceAB has also been observed in T4 and Tupedo strains in presence of vancomycin [94]. No study so far has shown a link between the BceAB and the BceRS in *S. pneumoniae*, it is supposed that they cooperate as seen in their homologues from other species.

1.6. Challenge of working with membrane proteins

Membrane proteins pose several difficulties regarding their handling. The first challenge is their expression. It is necessary that the expressing bacteria use similar amino-acid codons for optimal expression. Improper codons slow expression down. Additionally, over-expression of membrane proteins might be toxic to bacteria and might lyse the cells prematurely.

Another challenge is purifying and handling the protein after expression. Without a hydrophobic agent, such as a detergent or other apmhiphile, in the buffer solution, the protein tends to aggregate due to hydrophobic helices which congregate together. The common solution is solubilizing the membrane protein and separating it from the membrane using detergents forming protein-detergent complexes replacing protein-protein and protein-lipid complexes. The downside of detergent solubilization is that the protein is sometimes inactivated [188].

Protein extraction from membranes requires relatively high concentrations of detergents, which then may provoke protein instability. Nevertheless, it is necessary to keep the proteins soluble in aqueous solutions in order to perform *in vitro* studies of the membrane protein. Often the problem is resolved by lowering the detergent concentrations to limit the protein destabilization. Another solution may be transferring the protein to a less aggressive detergent or reconstitution of the protein in liposomes or amphipols. Alternatively, the membrane protein may be transferred to a nanodisc, which mimicks a cell membrane better than a detergent (Figure 1.26.).

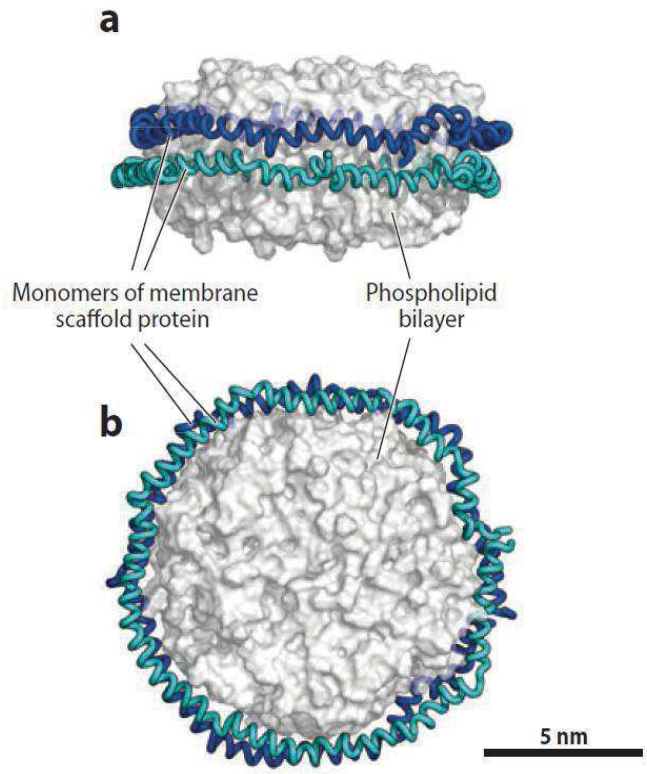


Figure 1.26. Molecular dynamic structure of nanodisc. A) Perpendicular and B) planar view of a nanodisc model. The membrane scaffold protein (MSP) forms two rings around high-density lipid bilayer [188].

Nanodiscs were first used by Sligar *et al.* [8, 202] and consist of 130-160 lipids organized in a bi-layer and surrounded by stabilizing proteins called membrane-scaffold proteins (MSP) (Figure 1.26.). To use nanodiscs, it is crucial to optimize the lipid-protein ratio. The diameter of the nandisk varies depending on the MSP. The advantage of nanodiscs lies in their versatility. The membrane protein embedded in them then may be studied in electron microscopy [77], solid-state NMR [124], small-angle X-ray scattering [133] etc. Another advantage is to study the function of the membrane proteins. Since the nanodisc simulates a cellular membrane, the protein is more likely closer to its native state than in detergent (detergents form micells around the protein), which may make activity measurements more reliable than measurements in detergents [188, 202].

2. Scope of the thesis

At the beginning of my PhD project the exact functioning mechanism of the BceAB-RS transporter complex was relatively poorly understood. Many studies concentrated on the functioning of the complex in *B. subtilis* and *E. faecalis*, which are considered as models. We were interested in the functioning mechanism in *S. pneumoniae*, as it might ultimately shed light on its resistance towards other antibiotics than AMPs.

The questions included: What AMPs does the complex transport? How does the BceAB type ABC transporter communicates with the BceRS type TCS? What role does the complex play in host-pathogen interaction? What is the stoichiometry of the complex BceAB transporter? What is the stoichiometry of the whole complex? How does the BceAB sense the AMPs?

To tackle these questions it was necessary to use many different approaches both *in vivo* and *in vitro*. *In vivo* part requires us working on the pathogenic *S. pneumoniae* D39 and creating mutants in order to assess the importance of the complex for the viability of the bacteria in various conditions growing the bacteria in liquid broths containing different AMPs. *In vitro* part of the work requires expression and purification of the four components of the complex. Since two of the components of the complex are membrane proteins, the TMD (BceB) of the BceAB transporter and the HK (BceS) of the BceRS, much time was spent on optimization of expression, solubilization and purification of the proteins. Due to time constraints I concentrated my efforts primarily on the BceAB complex, because this type of ABC transporter has not been described in detail yet. We studied its activity as well as complex stoichiometry.

Part 2. Results and discussion

Sommaire – Résultats et discussion

D'abord nous avons étudié le fonctionnement du système ABC-TCS type BceAB-RS *in vivo*, directement dans la souche D39 du pneumocoque. Nous avons délété les gènes du transporteur ABC type BceAB, et du TCS type BceRS et la double délétion ABC-TCS. Dans les conditions normales, il n'y avait pas de différences entre les mutants et la souche sauvage (WT). Nous n'avons pas observé de différence importante entre les souches pendant les expériences d'infections des mouches. Nous avons réalisé des courbes de croissance en présence des différents AMPs dans les souches de délétion. Nous n'avons pas observé une différence de croissance entre les souches en absence des AMP et en présence des AMP LL-37, ramoplanine et duramycine. Cependant nous avons découvert une différence importante entre les mutants et le WT en présence de bacitracine, nisine et vancomycine. De plus, il s'agit de la première étude démontrant un lien entre le BceRS et BceAB dans le pneumocoque par des expériences de qPCR. A partir d'une exposition de 15 minutes de la bacitracine sur le pneumocoque à, l'expression du BceAB est augmenté de 200 fois dans la souche sauvage (WT). Cependant cette augmentation n'est plus observée lorsque le BceRS est absent (souche ΔBceRS).

En parallèle, nous avons étudié les composants du système BceAB-RS *in vitro*. Nous avons d'abord exprimé et purifié le régulateur de réponse cytoplasmique (BceR) et nous avons essayé de le cristalliser sans succès. Nous avons aussi exprimé et purifié la kinase d'histidine (BceS). Malheureusement, les quantités étant trop faibles, nous n'avons pu continuer des études approfondies de cette protéine.

Malgré des problèmes au cours du clonage, des expressions et purifications du transporteur ABC type BceAB, nous avons réussi à le purifier en quantités suffisantes pour les futures expériences pour sur ce système. Les expériences d'activité d'hydrolyse de l'ATP par le BceAB et les mutants nous ont démontré que nous avons purifié une protéine fonctionnelle. De plus, nous avons aussi réussi à l'intégrer dans un nanodisc. Dans ce système, le transporteur présente une forte augmentation d'activité en comparaison de l'activité en détergent.

1. Studies of BceAB-RS type ABC-TCS system in *S. pneumoniae*

1.1. Preparation of *S. pneumoniae* strains

To knock out the BceAB ABC transporter (*Spd0804* and *Spd0805* in D39, corresponding to *Sp0912* and *Sp0913* in TIGR4) out of the *S. pneumoniae* D39 genome (ΔBceAB strain), we produced three separate PCR fragments: F1 and F3 were identical to the pneumococcal genes flanking the BceAB gene upstream and downstream respectively and F2 fragment contained a chloramphenicol resistance insert (CAT) (Figure 2.1. A).

The theoretical size of the F1 (869), F2 (1121) and F3 (849) fragments appeared at the correct size on the agarose gel. The F2 fragment contains two ~ 20 base overhangs which are identical to either the F1 or the F3 ends. To create the final F4 fragment (Figure 2.1. B) the individual F1, F2 and F3 fragments were purified, then hybridized together by PCR as described in the materials and methods. After F4 purification, we transformed the bacterial genome of the *S. pneumoniae* D39 by the cassette and selected transformed clones by growing the bacteria overnight on Columbia Blood Agar plates containing chloramphenicol. A PCR test directly on the clones using the forward primer of F1 and reverse primer of F3 resulted in 10 positive clones containing the CAT insert (Figure 2.1. C). With another set of outlying primers we amplified again the inserted fragment of clones 1, 2 and 3 and sent the samples for DNA sequencing (*Beckman Coulter Genomics*). The sequences corresponding to the F1 and F3 fragments were identical to the genomic DNA and the sequence corresponding to the CAT insert was identical to the cassette in the plasmid.

For the deletion of the BceRS TCS system (*Spd1445* and *Spd1446* in D39 corresponding to *Sp1662* and *Sp1663* in TIGR4), the same method was used, but a kanamycin resistance cassette was used instead creating the ΔBceRS strain. For the double knock-out, first the BceAB was knocked out and then the BceRS (ΔDouble).

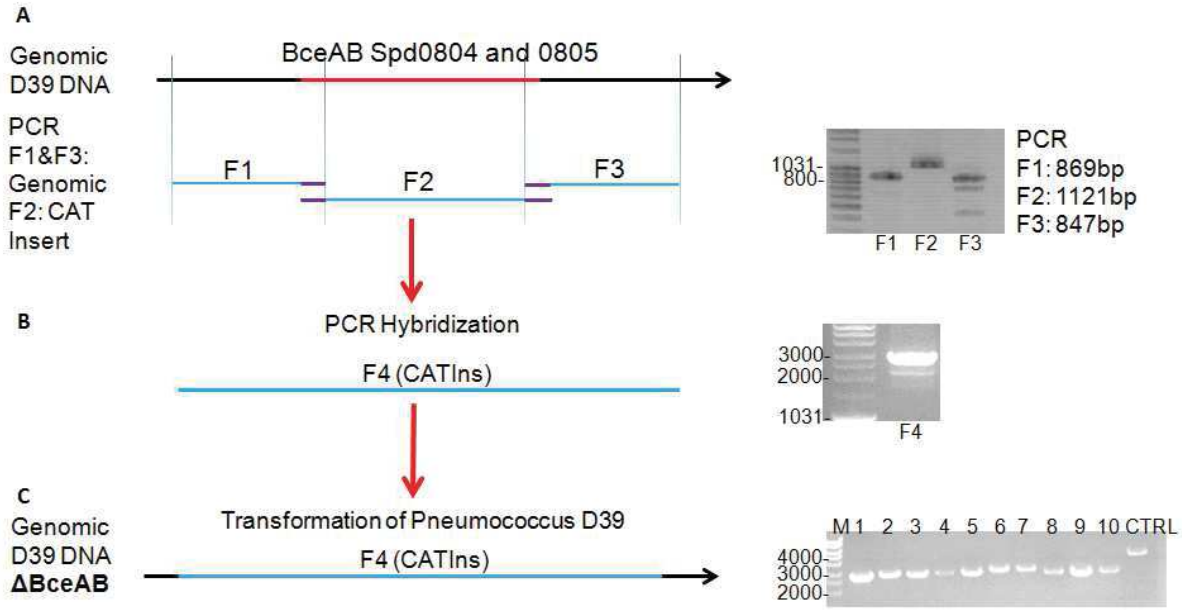


Figure 2.1. Preparation of *S. pneumoniae* D39 ΔABC mutant. A) Genomic representation of the NBD and TMD. Three fragments were prepared using PCR: F1 (869 bp) and F3 (847 bp) are identical to the pneumococcal genome flanking the BceAB type ABC transporter upstream and downstream respectively. F2 fragment (1121 bp) is a chloramphenicol insert with ~ 20 nucleotide ends identical to the ends of eithe F1 or F3, represented by a purple band. B) By PCR hybridization we created F4 fragment (2791 bp). C) After pneumococcal transformation, where the original genome was potentially replaced by the F4 insert, we grew the bacteria on a Columbia blood agar plate containing chloramphenicol. Then we tested the size of the insert from the clones using the forward primer of F1 and the reverse primer of F3. M- marker, 1-10 clones, CTRL- control sample of D39 WT.

Next, we verified the phenotype and viability of the created ΔBceAB, ΔBceRS, and ΔDouble strains. We first observed them under 400x magnification of a bright field light microscope (Figure 2.2. A-D). The shape of the wild type is a normal ovococcus and the predominant arrangement is a diplococcus. All three mutant strains appeared of the same size and shape as the wild type. They were also predominantly found in a diplococcus or short chains like the wild-type, but unlike the wild-type, the ΔBceRS had almost no short chains and only single cells or diplococci. This difference may have been caused by uneven pressure applied on the glass during sample preparation suggested by the unidirectional orientation of the bacteria. Nevertheless, no obvious deviation has been observed between the mutants and the wild-type bacteria.

We next verified the presence of the capsule on the different strains. It has been observed before that during transformation, the pneumococcus may lose the capsule and therefore virulence, sometimes virulence may be lost even during storage in the freezer [140]. We therefore performed experiments to verify the presence or absence of the capsule (Figure 2.2. E). We repeated the experiment three times at different times and the capsule sizes were:

WT 9.5 mm ± 1.3, ΔBceAB 7 mm ± 1.0, ΔBceRS 8 mm ± 1.5, and ΔDouble 7.3 mm ± 1.3. The control strain D39 CpsD lacking the capsule displayed sediment between 2.5 mm ± 0.5. Compared to the wild type, we observed that the mutants have kept their capsule through the genomic transformation. It is not uncommon for the clones to lose the capsule as seen in the past in the laboratory. It is therefore necessary to perform this experiment when manipulating the genome of virulent pneumococcus.

The next experiment to verify the viability of the strains was to record a growth curve. As seen in Figure 2.2. F, we perceived that all four strains grew in a highly similar manner. At about 2.5 hours the lag phase moved to the exponential phase and after reaching the stationary phase in 5 hours, the OD lowered as the bacteria began to lyse. The mutants did not exhibit a viability or phenotypical difference from the wild type *S. pneumoniae* D39 when grown in *in vitro* conditions. This suggests that the mutants did not lose their viability compared to the wild type and we may continue our experimental work.

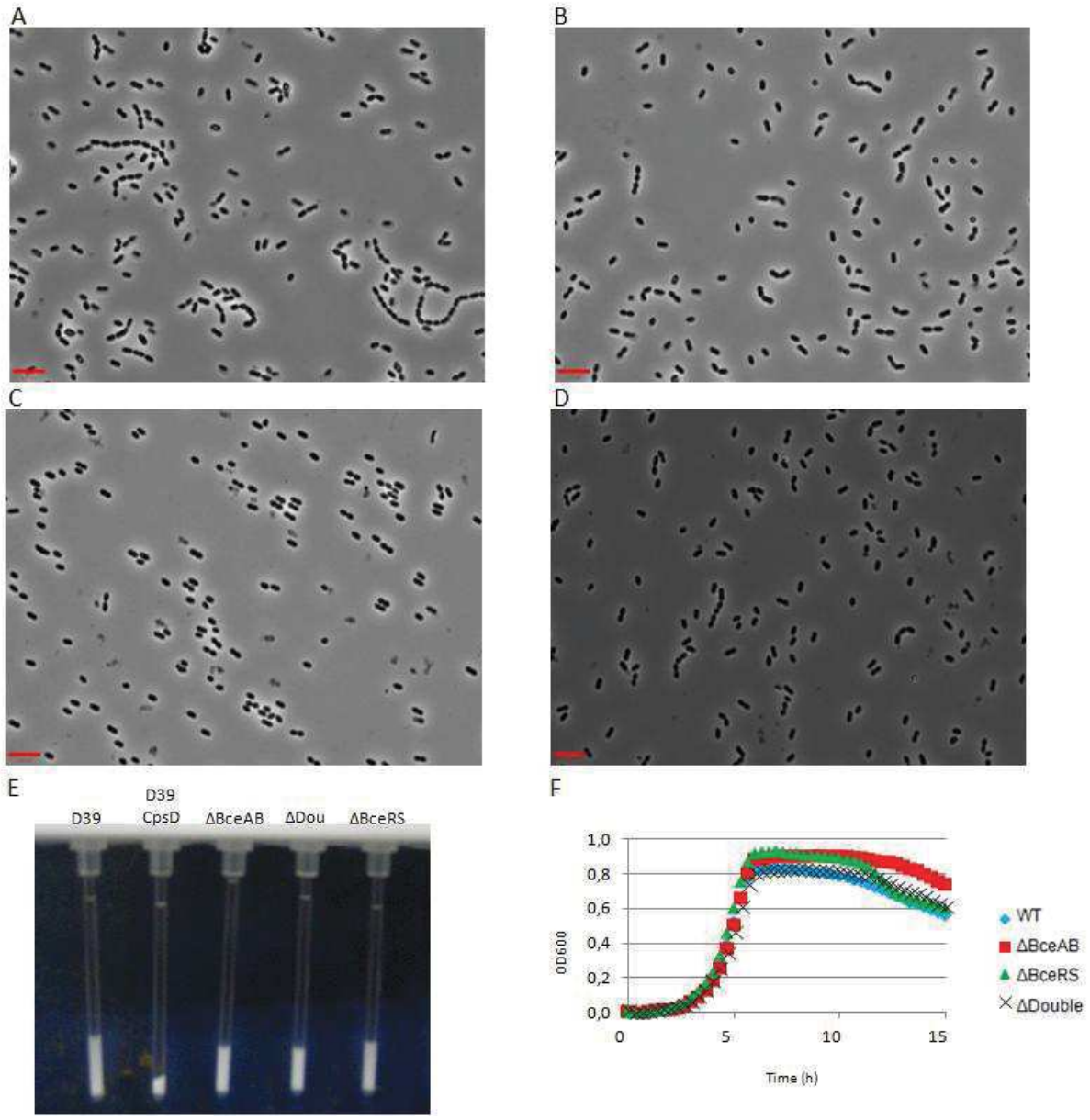


Figure 2.2. Control experiments of *S. pneumoniae* D39 WT and mutants. A)-D) 400x light microscope pictures of the **A)** Wild-type, **B)** ΔBceAB mutant, **C)** ΔBceRS mutant and **D)** ΔBceAB/BceRS mutant (ΔDouble). The basic shape of the bacterium is a ovococcus in all four cases and the preferred arrangement is either a short chain or diplococcus. **E)** A capillary experiment to investigate the capsule size as the wild type one. The WT and the mutants apparently kept a capsule of a similar size as the wild type one. A pneumococcus D39 CspD mutant lacking the capsule has been included for comparison. **F)** Growth curve of the *S. pneumoniae* D39 WT and mutants over 15 hours in THY media.

1.2. Pneumococcal BceAB-RS type ABC-TCS system does not play a role in host-pathogen interaction *(These experiments we performed in collaboration with Marie-Odile Fauvarque, CEA Grenoble)*

The next experiment we performed was to test *in vivo* the role of the BceAB-RS type ABC-TCS complex in the host-pathogen interaction using drosophilae as a model. In the past, several studies have shown the possibility to infect drosophilae with *S. pneumonia*, where bacterial virulence may be measured by drosophilae mortality. Additionally, the insect mounts an impressive AMP response [1, 185], which is relevant to our subject of study. Another advantage of this model is the availability of mutants lacking a good immune response and AMPs, such as ΔTAK1 mutants [126]. We therefore chose to use the drosophilae as a model to the relative ease of handling and low cost compared to other animal models. Theoretically, we would expect the mutant strains to be less virulent than the wild-type as it may lack resistance towards insect AMPs.

The presented data is a representative example of four different experiments. The flies were infected by the pneumococcus and its mutants by pricking with a needle dipped in 50 µl of 1.3 x 10^{10} cfu/ml ± 0.15 of bacteria (Figure 2.3.). Several flies of the control, non-infected (NI) group did not survive the injection, but more than 80% of them survived three days post infection. Flies infected by the wild type began dying after 20 hours post infection. Within 68 hours post infection, only 8% of the flies survived the pneumococcal infection. Flies infected by the mutants also began dying after 20 hours post infection similarly to the wild type. At about 30 hours flies infected by strains followed a similar death rate. At 68 hour post infection all infected flies had about 10% survival rate, showing no major difference between the wild type and the mutants, despite the slightly higher death rate of ΔBceAB infected flies in this experiment. This data corresponds to previous works, where drosophilae infected with lethal doses of *S. pneumoniae* SP1 strain died within two to three days post infection [185].

In other experiments, ΔBceRS, ΔDouble, or the wild-type showed different mortality even though the same conditions were followed; therefore it is precarious to interpret the results to a well-founded conclusion. Nevertheless, the impression is that neither the BceRS, nor the BceAB play a role in host pathogen interaction for the drosophilae model. Therefore, we may hypothesize that the system probably does not transport AMPs which are produced by this insect.

It would be interesting to use another model, such as the mouse, which is more closely related to humans than drosophilae, the AMPs produced by mice are also more closely related. Also it would be possible to test the difference in virulence between the wild-type and

the mutant strains in different types of infection, such as nasal infection, blood borne infection etc.

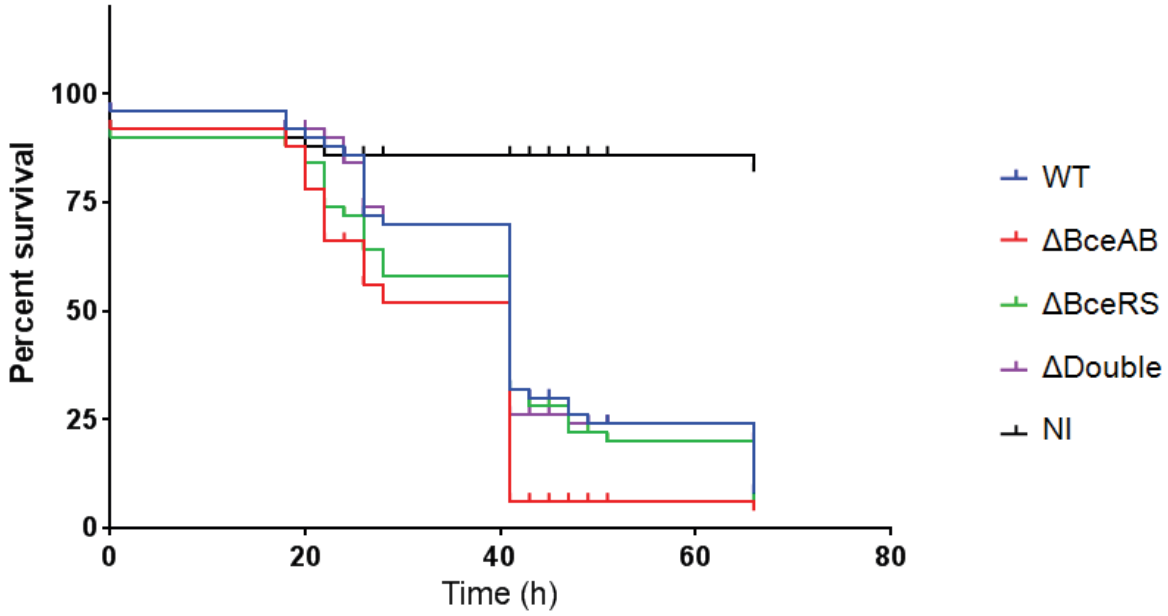


Figure 2.3. Survival of drosophilae after infection by *S. pneumoniae* D39 and mutants. 50 flies per condition were infected by pricking in the abdomen under the wing with a needle dipped in PBS containing 1.3 x 10^{10} cfu/ml ± 0.15 bacteria. After infection, the flies were kept with ample nourishment at 25°C for 18 hours before being placed at 30°C. Control flies (NI) were only pricked with a sterile needle. Time points when dead drosophilae were counted are marked with a tick or a line break.

1.3. Both the BceAB and the BceRS of pneumococcal BceAB-RS type system are necessary in resistance towards some AMPs

Next, we tested the growth of the bacteria in presence of AMPs, which were known to induce at least one of the three BceAB-RS type ABC-TCS complexes in *B. subtilis* [218]. In the absence of AMPs, the growth curves superposed on each other and no difference was observed between the wild-type and the mutants (Figure 2.4. A) as shown before. We then performed a series of experiments with various concentrations of different AMPs, where we expected to observe a difference between the wild-type and mutant strains in some cases. We selected six commercially available AMPs which were used in a study by Staron *et al.* in 2011 [218] of the three BceAB-RS type ABC-TCS systems in *B. subtilis*.

We present a typical concentration for each AMP tested, which is a compromise between a concentration without an impact on bacterial growth and a higher concentration killing all four strains. From the growth curve, it is possible to calculate the rate at which the bacteria divide (Figure 2.4.). When grown in normal THY liquid broth, the WT and mutants grew at a normal rate of about 1.5 generations per hour, giving a forty minute doubling time.

In presence of the peptide bacitracin at 2 µg/ml the wild type as well as the other three strains did not display any growth (data not shown). At the concentration of 1 µg/ml the growth curve and rate of the wild type strain was comparable to the growth curve and rate without any AMP, yet somewhat delayed. The difference in growth without AMP and in presence of bacitracin was not statistically significant (Figure 2.4. B and H). Compared to the wild type, all three deletions showed a severe growth inhibition in the same manner. The growth phase was severely delayed and the growth did not reach the standard OD. Additionally, the growth rate is three times lower than in the case of the wild type or in the absence of AMPs. It has been shown in the past that the BceAB transporter from the pneumococcus (R6 and D39 strains) was involved in the resistance towards bacitracin, where the susceptibility towards bacitracin of the ΔBceAB mutant (MIC 1 µg/ml, correlating with our result) was almost four times higher than the wild type (MIC ~ 4 µg/ml) [12, 150], however the involvement of the BceRS system has not been revealed for this bacterium. The fact that no difference is seen between the growth of the two separate deletions, ΔBceAB and ΔBceRS, and the double deletion ΔDouble, suggests that both the ABC transporter and the supposedly regulating TCS are necessary to confer resistance towards bacitracin as seen in *B. subtlilis* [59, 173, 201] and *E. faecalis* [71]. Bernard *et al.* in 2007 [13] showed in *B. subtilis* that the lack of function of either component of the BceAB type ABC transporter or the BceRS type TCS increases the susceptibility towards bacitracin.

When the bacteria were challenged with various concentrations of LL-37, the wild type did not show any susceptibility up to the concentration of 50 µg/ml, which was quite surprising as the MIC has been determined previously at 14 µg/ml for the D39 strain [150]. Additionally, no difference was seen between the wild type and the deletion strains in presence of LL-37, which is a confirmation of a result seen Majchrzykiewicz *et al.* in 2010 [150]. Interestingly, the same study shows a slight increase in expression of the BceB upon LL-37 challenge, but not of the BceA. It might be plausible that the pneumococcal TMD could bind LL-37 as one of the homologous BceAB-RS type ABC-TCS systems in *B. subtilis*, YxdJK-LM, whose only known substrate is LL-37 [218]. In the pneumococcus, another TCS system, TCS03 (*Sp0386* and *0387*) was slightly overexpressed when the bacteria was challenged by the AMP [150]. We may speculate that the BceAB-RS type ABC-TCS in the pneumococcus only transports some bacterial AMPs produced by its competitors such as *S. epidermidis* or *S. salivarius* and not eukaryotic AMPs, since we have not observed a difference between the mortality of the wild-type or the mutant strains in drosophilae infections. Yet, more data are necessary to really support this claim.

The presence of duramycin demonstrated a comparable response as LL-37. The wild-type pneumococcus did not show any susceptibility up to the concentration 100 µg/ml. Similarly, the mutant strains behaved as the wild-type did in presence of duramycin (Figure 2.4. G). In the past, the drug has been shown to be ineffective against *B. subtilis* and no involvement of the BceAB-RS type ABC-TCS system has been shown [218].

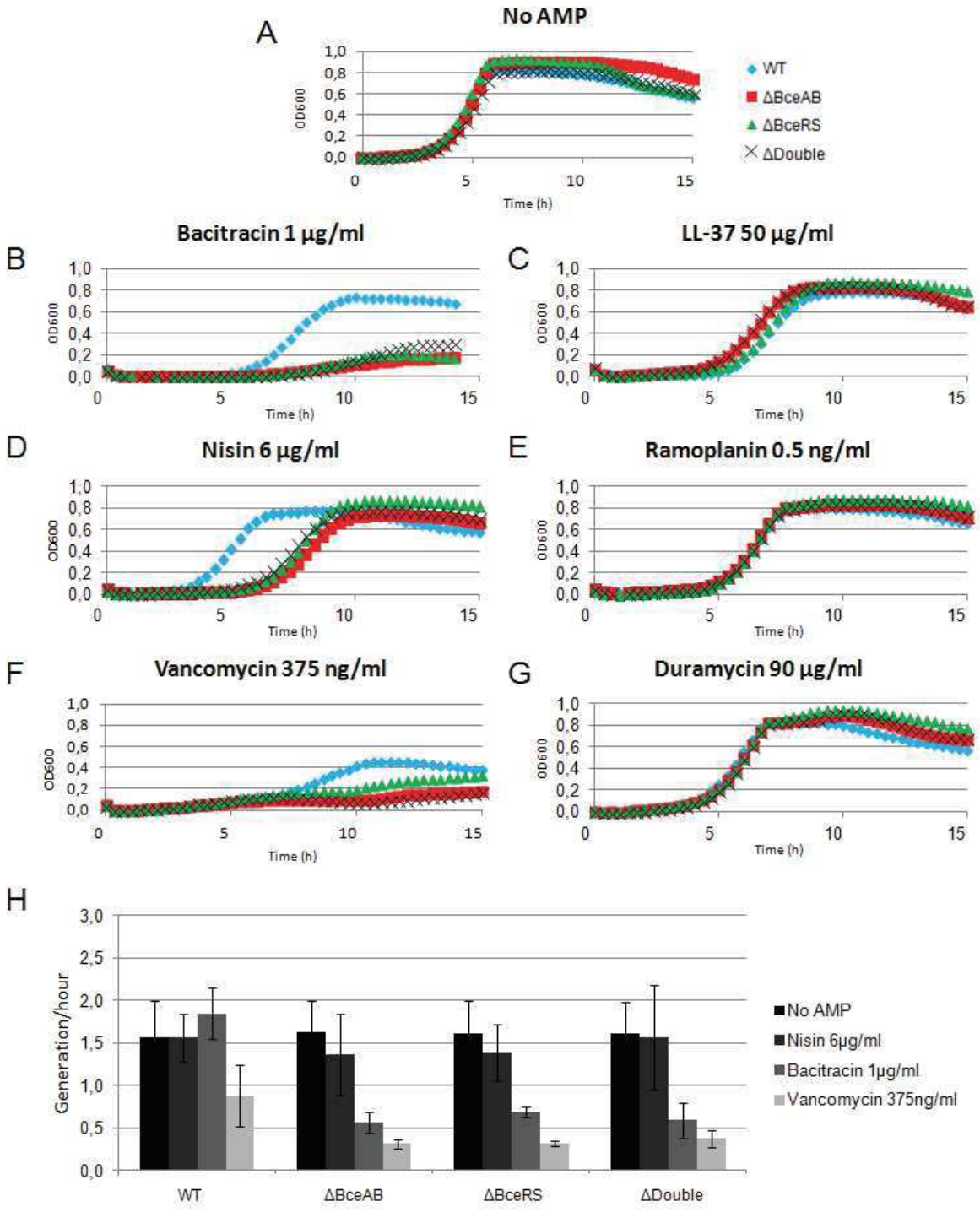


Figure 2.4. Growth curves and rates of *S. pneumoniae* D39 WT and mutants in liquid broth in presence or absence of AMPs. Bacteria were grown to OD_{600nm} of about 0.3. They were then centrifuged and resuspended in 1ml of THY and the OD_{600nm} was re-measured. For the OD_{600nm}= 0.3, 36 µl of the bacteria were placed in 15ml of THY. Solution with bacteria (200 µl) was then placed into a well containing 100 µl of THY with or without AMPs. The OD_{600nm} of the wells in the plate were then measured every 20 minutes for 15 hours using FluoStar (BMG Labtech). The presence and the concentration of a given AMP are indicated above the corresponding growth curves. The growth curves were done in triplicates and performed at three separate times. H) Growth rates were calculated using the log scale of the growth curve where the transition from lag phase to exponential growth forms a straight line. The slope (m) of a line is used in the following equation: doubling time = $1/(\ln(2)/m)$.

The presence of 6 μg/ml of nisin does not seem to affect growth rate of the wild-type *S. pneumoniae* D39 (Figure 2.4. D and H). The growth curve also appears equal to the one when grown without AMPs without any delays. On the other hand, the presence of nisin delayed the growth of all three mutant strains compared to the wild type. It has been previously shown that the BceAB transporter in the pneumococcus was involved in nisin resistance [150], but the involvement of the BceRS has been unnoticed to this day as in the case of bacitracin. Staron *et al.* in 2011 [218] demonstrated in *B. subtilis*, that a BceAB type homologue PsdAB is responsible for nisin resistance, and entirely dependent of the BceR type RR homologue, PsdR using mutation analysis. The involvement of a TCS system in resistance towards nisin in a BceAB-RS type ABC-TCS homologue was further confirmed in another bacterium, *S. aureus* in 2013 by Kawada-Matsuo [121]. Contrary to these results, the two BceAB type ABC transporters and the BceRS type TCS in *E. faecalis* are not involved in nisin resitance [84].

Remarkably, the growth rate for all four strains is roughly similar to the growth rate without AMPs (Figure 2.4. H). The delayed but normal growth rate by the mutants may be explained by a longer period of adaptation of the bacteria to nisin presence by other proteins responsible for nisin resistance. It has been previously demonstrated that mutations in *dlt*A, which is normally responsible for D-alanylation of teitoic acids, also increase sensitivity towards nisin [129]. Also another TCS system, specifically TCS03 as in the case of LL-37, has been shown to be involved in nisin resistance in the TIGR4 strain and is overexpressed in its presence [123]. We may yet conclude that both the BcetAB-RS type ABC and TCS are necessary for a timely onset of nisin resistance in the pneumococcus.

The AMP ramoplanin presented a formidable challenge to the growth of the wild type pneumococcus. It killed the bacteria at a concentration as low as 0.3 ng/ml, which makes it the most powerful of the tested AMPs. The mutant strains showed equal susceptibility to the drug as the wild type (Figure 2.4. E). None of the known homologues in the *B. subtilis* showed involvement in resistance towards ramoplanin either [218], but the bacteria grew even at the concentration of 5 μg/ml. It is interesting to note that the AMPs ramoplanin and nisin have the same mode of action by binding to lipid II and membrane pore formation, yet it is only in the presence of nisin that the wild-type and the mutants displayed a difference in growth. The determining factor might be the shape of the AMPs, because ramoplanin is circular and nisin is more linear containing several loops. Nevertheless, even structurally close molecules may not be recognized by the same BceB type TMD [83, 218].

Introduction of vancomycin to the media presented a challenge to the growth of all four strains (Figure 2.4. F). While the wild type managed to slowly grow to OD_{600nm} of approximately 0.5, none of the three mutants reached that value. Plus, all strains displayed slow growth in presence of 375 ng/ml of vancomycin. Compared to the wild-type, the mutants did not reach such a high OD and the growth curve did not show the typical lag, exponential and stationary phases.

In addition, their growth rate was also significantly lower regardless of the mutation. It has been shown in the past that other TCS systems were overexpressed in resistant pneumococcal strains upon vancomycin challenge, specifically TCS03 and TCS11 (*Sp2000* and *Sp2001*) [94]. Nonetheless, Kietzman *et al.* [123] claims that vancomycin does not induce TCS03 expression. Haas *et al.* [94] also observed an overexpression of the BceAB type ABC transporter in the TIGR 4 strain upon vancomycin stress, but did not describe its involvement in the resistance towards the AMP.

All three mutant strains react in the same manner to challenges by many AMPs, indifferently of the differences or similarities in the structures and composition of the AMPs. Interestingly, in *B. subtilis*, the resistance towards nisin and bacitracin is executed by two homologous BceAB transporters and vancomycin isn't transported by any of the three homologues present in its genome [218]. It has been demonstrated previously that structurally unrelated AMPs are recognized by the large BceB extracellular domain, while related AMPs did not bind to the BceB subunit at all [83]. Consequently, despite the fact that bacitracin, nisin and vancomycin have different structures and modes of action, as described in the introduction of this manuscript, it is not uncommon that they would bind the same pneumococcal BceB subunit. Nevertheless, both bacitracin and nisin have different structures; both use UPP as a primary docking site for antimicrobial activity. Vancomycin, apparently, has nohing in common structurally with the other two AMPs and its antimicrobial action is inhibiting cell wall trans-glycosylation and trans-peptidation, which is quite puzzling given the action site of the AMP [200] and the position of the BceAB in the membrane.

We may therefore conclude that for resistance towards bacitracin, nisin and vancomycin, the whole BceAB-RS type ABC-TCS system is necessary. The presence of one of the components of the system, being the ABC or the TCS is not sufficient to provide even partial resistance towards the AMPs. Therefore, this supports the assumption of the cooperation of the BceAB type ABC transporter and BceRS type TCS to afford a significant level of resistance to some AMP.

1.4. BceRS directly regulates BceAB expression upon AMP introduction *(Experiments performed by C. Durmort and L. Bellard)*

To study further the relationship *in vivo* between the ABC and TCS of the BceAB-RS system we decided to use qPCR and observe the induction of ABC expression upon stress by bacitracin. It has been shown previously in *B. subtilis* that BceAB overexpression is induced by this AMP [201]. In the following experiment wild-type bacteria and ΔBceRS mutant were grown in presence of bacitracin for 30 min and the mRNA levels were measured at 5, 15 and 30 min as above.

For the wild-type strain we observed a 47-fold (± 27-fold) and 40-fold (± 22-fold) increase of mRNA expression of the BceB and BceA respectively already at 5 min after bacitracin challenge (1 µg/ml). At 15 min the BceB was being expressed 166-fold (± 97-fold) higher than at T0 and BceA 116-fold (± 24-fold). At 30 min the BceB expression was 210-fold (± 31-fold) higher and BceA 159-fold (± 30-fold). All of the other genes which were studied, the BceS and BceR of the TCS and PatA, a part of an MDR transporter involved in acriflavine, ciprofloxacin, and norfloxacin resistance [24] as a control, did not display a modification of expression level upon bacitracin stress (Figure 2.5. A).

Interestingly, Majchrzykiewicz *et al.* in 2010 [150], observed only a 12-fold and 8-fold increase in expression of the BceB and BceA, respectively, in 15 min and 10-fold and 9-fold increase of expression in 30 min in presence of 0.7 µg/ml of bacitracin. This difference might be explained by a different experimental approach, as they used β-galactosidase assay. In *B. subtilis*, similar values as ours of induction of the BceA and BceB were observed by Rietkotter *et al.* in 2008 [201] when the bacteria was challenged for 30 mins between 3 µg/ml to 300 µg/ml of bacitracin: the expression was increased 100-fold to 160-fold, respectively. Additionally, the BceAB-RS system was shown to be particularly sensitive to bacitracin presence, where induction of BceAB expression was observed already at 0.03 µg/ml.

While we observed tremendous expression of the mRNA of the ABC components in the wild type in presence of bacitracin, the expression of the BceAB type ABC transporter remained unchanged for the ΔBceRS mutant. This result is congruent with previous results in *B. subtilis* [13], *E. faecalis* [84] and *S. aureus* [121] where the presence of the BceRS type TCS is obligatory for BceAB transporter expression and thereby resistance towards bacitracin.

To our knowledge, this is the first report to demonstrate that the BceRS type TCS regulates the BceAB type ABC transporter expression upon bacitracin stress in *S.*

pneumoniae. Further experiments are needed to observe if the same overexpressing phenomenon occurs in the presence of nisin and vancomycin.

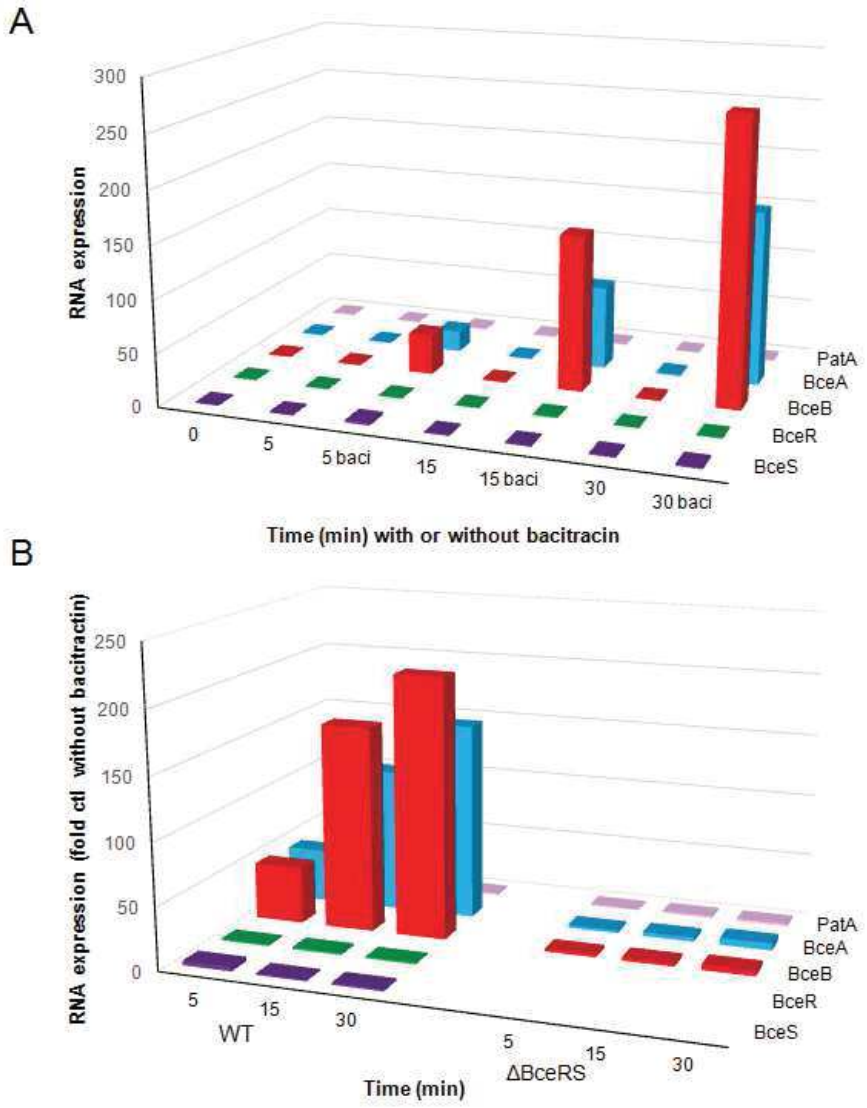


Figure 2.5. qPCR experiments of *S. pneumoniae* WT and ΔTCS in absence or presence of bacitracin. mRNA measured in this experiment are PatA as a control, which is not affected by the presence of bacitracin. Genes of interest were BceB (TMD) and BceA (NBD) of the BceAB type ABC transporter and the BceS (HK) and BceR (RR) of the BceRS type TCS. The bacteria were grown to the OD_{600nm}=0.3 before introduction of the AMP (T0). **A)** Fold expression of the components of the ABC-TCS system against T0. **B)** Fold expression of BceAB-RS type ABC-TCS system components at 5, 15 and 30 minutes in presence of bacitracin vs. the same time point without bacitracin for the WT and ΔBceRS mutant.

2. Functional studies of the pneumococcal BceAB-RS type complex

2.1. Purification of the individual subunits comprising the BceRS type TCS

We first expressed the BceR (RR) of the BceRS type TCS, as it is a soluble protein. We performed the expression in BL21(DE3) cells transformed by the pET-Duet-1-RR plasmid. As seen in Figure 2.6.A, there was a leak of BceR expression even before IPTG induction, however the expression was increased by IPTG. On the Western blots we observed a band at ~ 25 kDa, which corresponds to the BceR theoretical size of 26 kDa. After cell lysis, we tested the expression solubility of the protein by centrifugation (Figure 2.6. B). The majority of the expressed BceR was in inclusion bodies, but a substantial amount was left in the supernatant, leaving enough material to work with further. We then performed a Ni-NTA affinity chromatography purification immediately followed by a size exclusion chromatography. The first peak at ~ 373 ml is most likely aggregates, or maybe DNA-bound protein. The second small peak at about 381 ml is probably a dimer of BceR as the size is ~ 65 kDa. The large peak at about 395 ml likely corresponds to a BceR monomer with a size of ~ 30 kDa. (Figure 2.6. C and D). We were able to purify 42 mg of BceR from four liters of bacterial culture after pooling all fractions between 15 and 41. After concentration, an overloaded Commassie blue stain did not reveal contaminants (data not shown).

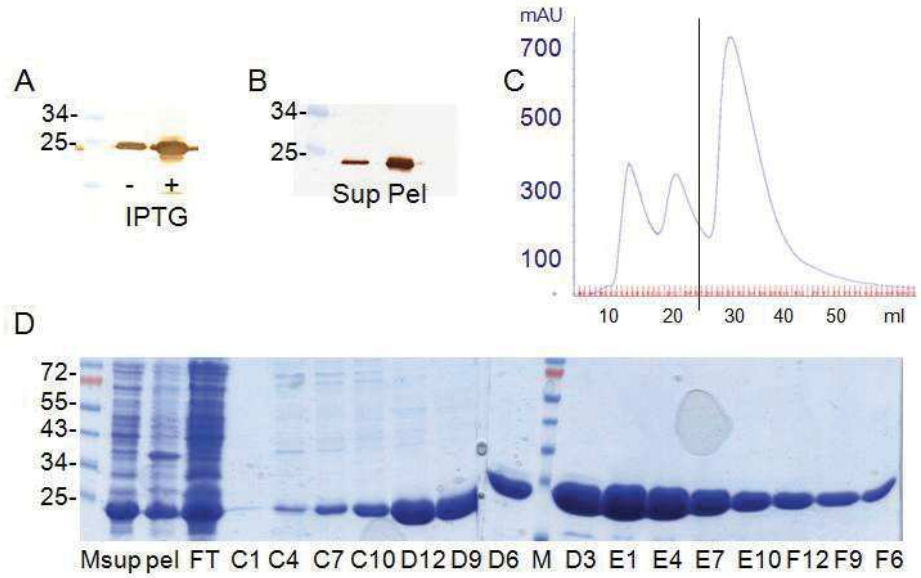


Figure 2.6. Overexpression and purification of the BceR. A) Western Blot of BceR overexpressed in BL21(DE3) before and after induction by IPTG. The bacteria were transformed by the plasmid pET-Duet1-RR and were induced (lane +) or not (lane -) by addition of 1 mM IPTG and further grown at 37°C for 6 h. B) Western Blot of the soluble and insoluble fraction of BceR. The cells expressing the BceR were lysed by sonication and the lysate was then ultracentrifuged at 100 000 g for 1h. Sup: supernatant, Pel: pellet C) After Ni-NTA column purification, the eluate of 4 ml containing the BceR analyzed by absorbance at 280 nm, was directly injected into 125 ml Superdex 75 size exclusion chromatography column. The graph represents the chromatogram obtained with the absorbance at 280 nm plotted versus the elution volume. The two red lines show the collected eluates (1 ml) beginning with fractions #1 to #41. D) Gel of the samples collected from gel filtration fractions: M: molecular weight markers (kDa), sup: supernatant (injected sample), pel: pellet, FT: flow through from Ni-NTA column purification, then every three fractions under the three larger peaks were selected to do the Coomassie Blue stained SDS-PAGE gel.

We next decided to pursue the study of BceR using high throughput crystallization screening platform available at the EPN science campus in Grenoble. We concentrated the protein to 12 mg/ml by serial 5 minute centrifugation at 20 000 g in a 10 000 MW CO PES column (*Millipore*) and froze aliquots at -80°C. Aliquots were then thawed, centrifugated and supernatant placed in fresh tubes before being sent for crystalography screening at 4°C. In none of the conditions the protein produced a crystal. We continued to pursue the crystallography experiments by making an additional 120 conditions at 4°C and 20°C, with the same negative result. Due to time constraints we decided to follow other parts of the project, but the next step would have been trying different protein concentrations and different precipitants.

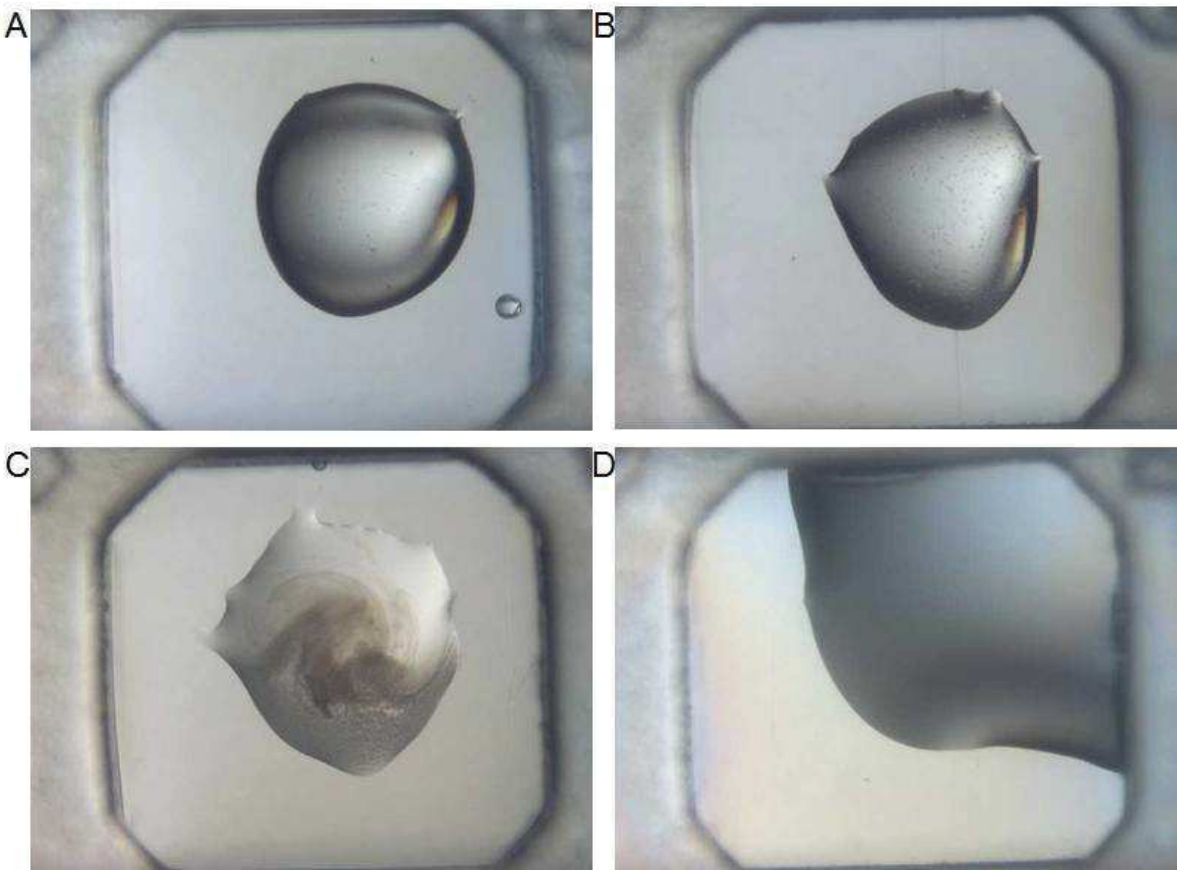


Figure 2.7. Crystallogenesis experiments of the BceR. The purified protein was sent to the crystallography platform (Highthroughput crystalogenesis platform at ESRF) and different screening plates were used (in total, 600 conitions were tested). Examples of hanging drops are shown here where 1µl of purified protein at 8 mg/ml was mixed with 1 µl of screening solution and left for 8 weeks at 4°C. None of the conditions tested produced any crystal. A) and B) crystals of salt and precipitated protein are visible in the drops. C) Protein precipitates (brownish colour). D) This solution remains clear.

Next, we pursued the expression and purification of the BceS (HK). We expressed the 38 kDa protein containing an *N*-terminal streptavidin tag in BL21(DE3) transformed by a pRSF-Duet1+HK plasmid. Upon overnight expression after IPTG induction only a small amount of protein has been expressed, since no bands have been observed in a SDS-PAGE gels stained by Coomassie blue stain or a silver stain (Figure 2.8. A). After cell lysis in the microfluidizer at 18 kpsi, we then separated the membrane from the rest of the cell lysate by ultracentrifugation (130 000 g). We next tested the protein solubilization by several detergents. We used 1% w/v detergent concentration for overnight solubilization (Figure 2.8. B). Despite the fact that lauryl maltose neopentyl glycol (MNG3) displayed a relatively low, but consistent solubilization efficiency we chose to continue working with this detergent as in later experiments we used MNG3 for solubilization of the BceAB transporter, and it also showed higher ATPase activity in this detergent. We then proceeded to continue the

purification process on a Strep-Tactine® column (*IBA*). We observed that much of the protein did not attach to the affinity column and therefore in addition to losses of the protein during solubilization, much material was lost during purification as well. We were able to visualize the protein only using α-streptavidin Western blotting using highly sensitive chemiluminescent substrates as during Coomassie blue straining or even silver staining no band was observed. Because of these difficulties and the delicacy of the Streptavidin resin, we tried to generate two new plasmids with a histidine tag on the BceS either on the *N*-terminus, or the *C*-terminus. Despite our efforts, we did not succeed in these new cloning strategies.

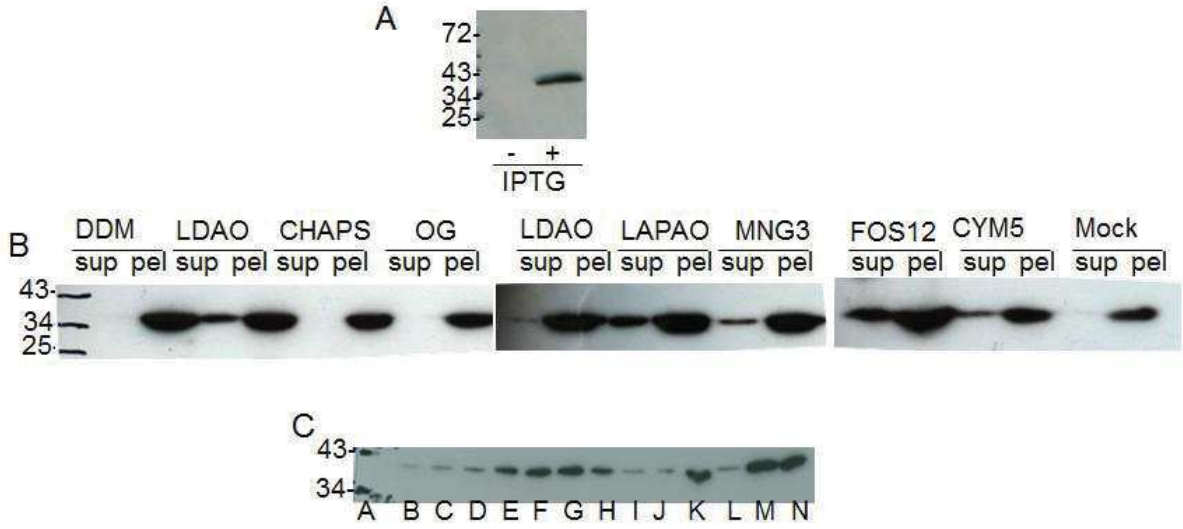


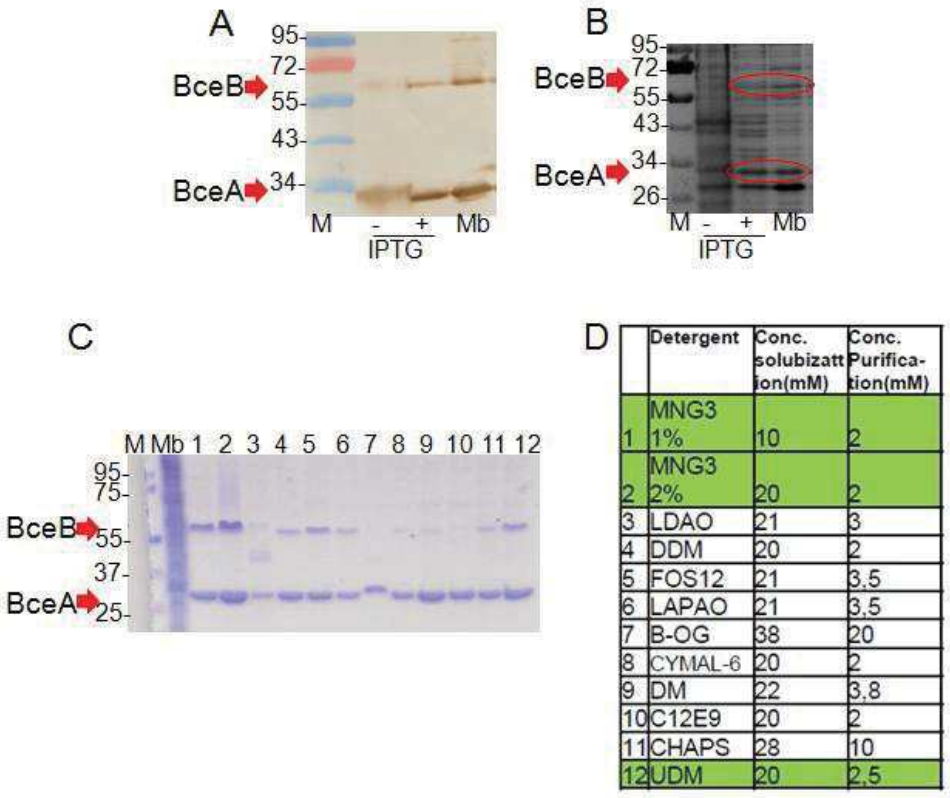
Figure 2.8. α-Streptavidin Western Blots of BceS overexpression, solubilization and purification. A) Total bacterial extract containing the overexpressed streptavidin tagged BceS. Bacteria BL21(DE3) were transformed by the plasmid pRSF-Duet1-HK and were induced (lane +) or not (lane -) by addition of 1 mM IPTG and further grown at 20°C over-night. B) Solubilization test of total membrane extract containing the BceS protein using different detergents. Sup: supernatant; Pel: pellet. C) Purification of BceS on a Strep-Tactin™ column after ON solubilization with 1% w/v MNG3 and purification in 0.1% of the same detergent. A- molecular weight markers (kDa); B-J – eluted fractions (7.5 µl sample loaded on gel form 1 ml eluate); K- flow through; L- wash; M- supernatant of solubilized membrane (5 µg); N- membrane before solubilization (5 µg).

2.2. Characterization of the purified BceAB type ABC transporter

We tested the expression of the BceAB type ABC transporter in several systems such as BL21(DE3), C41(DE3) and C43(DE3) [162], Rosetta2 (*Roche*) cells and the best yield of expression was obtained in the BL21(DE3). We then expressed the BceAB type ABC transporter overnight at 20°C. On the SDS-PAGE gels in Figure 2.9. A and B, a clear induction of expression upon IPTG introduction may be observed. The cells were then broken using the CellDestruptor at 18 kpsi and the membranes were separated from the rest of the

lysate using ultracentrifugation at 130 000 g for two hours. Even though the theoretical size of the tagged transmembrane subunit BceB is 75 kDa, it regularly migrated to about 60 kDa in SDS-PAGE gels. The BceA theoretical size is 28 kDa and its corresponding band displayed a slightly larger size of roughly 30 kDa. As a control of our protein expression, we chose to perform *N*-terminal sequencing of each subunit. For that purpose, we used FOS12 detergent for membrane solubilization. The detergent was very efficient, but presumably will denature the protein [249]. Nonetheless, in order to confirm the identity of both proteins by *N*-terminus sequencing, it was adequate. After Ni-NTA affinity column purification we performed *N*-terminus sequence analysis of the first eight amino-acids of each subunit (analysis done by Jean-Pierre Andrieu at the IBS) and the analysis confirmed the identity of our protein (BceB: MFRLTNKL, BceA: GHHHHHHH).

To separate the protein from the rest of the membrane it was necessary to screen for the best detergent to solubilize it. We used the ROBIOMOL platform, present at the IBS to screen for 11 detergents (Figure 2.9. C and D). The detergents giving the best yield of solubilization of the BceAB transporter were MNG3 and UDM. Since qualitatively MNG3 seemed more efficient and the BceS protein was also solubilized by the same detergent, we chose to pursue the study with MNG3. Additionally, in a previous study done in the group [249], it has been shown that the solubilization and subsequent purification of BmrA with MNG3 displayed a relatively low presence of contaminants along with high stability compared to other detergents. It has been shown also to increase the stability of other membrane proteins and may help to generate larger and better diffracting crystals [30].



	Detergent	Conc. solubizatt ion(mM)	Conc. Purifica- tion(mM)
1	MNG3 1%	10	2
2	MNG3 2%	20	2
3	LDAO	21	3
4	DDM	20	2
5	FOS12	21	3,5
6	LAPAO	21	3,5
7	B-OG	38	20
8	CYMAL-6	20	2
9	DM	22	3,8
10	C12E9	20	2
11	CHAPS	28	10
12	UDM	20	2,5

Figure 2.9. Overexpression and solubilization test of the BceAB type ABC transporter. A) and B) Western blot and Silver stained SDS-PAGE gels, respectively, of the BceAB transporter overexpressed in BL21(DE3). The bacteria were transformed by the plasmid pRSF-Duet1-ABC and were induced (lane +) or not (lane -) by addition of 1 mM IPTG and further grown at 20°C ON. Membrane (Mb) were extracted from lysed cells using ultracentrifugation. C) Coomassie blue stained SDS-PAGE gel of ROBIOMOL solubilization test of total membrane extract containing the overexpressed BceAB transporter by different detergents. M- molecular weight markers (kDa), Mb- membrane, 1-12 see D). Numbers in the far left column correspond to the numbers in C). Detergent abbreviation names are in the left column. Right column shows the concentration of the respective detergent used for solubilization of membrane aliquots at 1 mg/ml of total protein for 2 h at 4°C. Far right column shows concentration of detergent used for washing and elution on Ni-NTA affinity column.

To purify the BceAB type ABC transporter, we first solubilized the membrane typically at 4 mg/ml using 1.5% (w/v) MNG3 overnight at 4°C. We diluted the sample to 0.5% (w/v) MNG3 then centrifuged it at 110,000 G for 1h, and we incubated the supernatant with Ni-NTA resin for 4 h under agitation. After extensive washes in a buffer containing 50 mM imidazole we eluted the protein with 300 mM imidazole (Figure 2.10. B and C lanes C to J). It is important to note that in the Western blot, the signal of the BceA is higher than the signal of BceB, even though on the gel with Coomassie blue staining, the reverse is observed. It is therefore possible that the histidine tag on the BceB becomes somehow masked or may be less reactive with the antibody.

After the affinity column purification we performed size exclusion chromatography experiments. After several attempts to concentrate the transporter where we observed

consistently important precipitation of the protein, we chose to inject the eluate from the Ni-NTA purification with the highest concentration of protein into the column. The large peak on the chromatogram at 11 ml (Figure 2.10. A) corresponded to an apparent molecular mass of ~ 310 kDa, and is a complex of BceAB-detergent as we saw both subunits in the SDS-PAGE gels (Figure 2.10. A and B, lanes K and L). The small peak corresponds to an apparent molar mass of ~ 80 kDa, and may be a dimer of BceAs (Figure 2.10. B and C, lane M). Despite the fact that the peak which eluted at 11 ml in the size exclusion chromatography step is somewhat symmetrical, with a slight shoulder around 9 ml (Figure 2.10.), due to its large width (3 ml), dissociation of the complex is very likely. This was confirmed by protein analysis on line (PAOL) experiments where gel filtration is combined with light scattering (data not shown). The same pattern was observed for samples from other protein preparations at two different detergent concentrations, 0.01% and 0.005% (w/v) MNG3, regardless of protein sample concentration (between 1.5 and 3 mg/ml after nickel purification, and before gel filtration). These results indicate the reproducibility of the purification protocol; however at protein concentrations below 1.5 mg/ml, a higher level of dissociation and aggregation is apparent as more peaks appeared between 10 and 16 ml of elution.

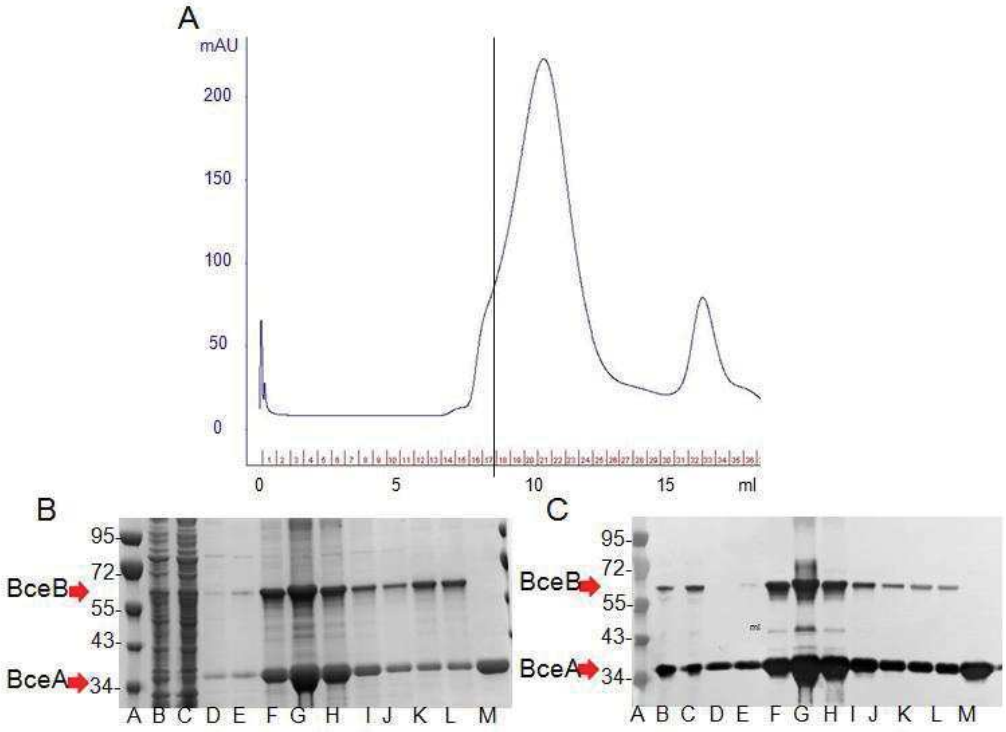
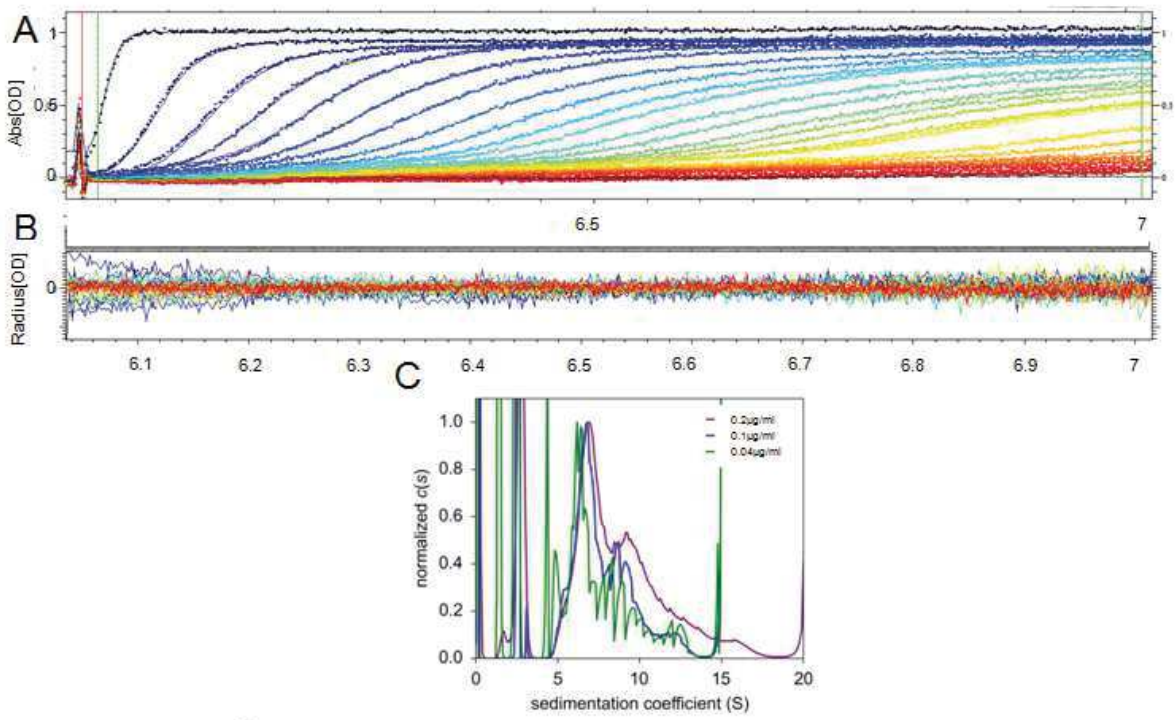


Figure 2.10. Purification of the BceAB ABC transporter. A) Graph representing a chromatogram obtained with the absorbance at 280 nm agains elution volume of the purified BceAB transporter. The protein was purified on Ni-NTA as seen in B) and C). The column used for gel filtration was Superdex S200 10/300 GL. The black line represents cut-off value of column dead volume. B and C) Coomassie blue stained SDS-PAGE gel and Western Blot of the ABC transporter purification on a Ni-NTA affinity and size exclusion chromatography columns. A- molecular weight markers (kDa), B- solubilized membrane, C- flow through, D- wash, E-J- Ni-NTA affinity eluates, K-L- size exclusion chromatography fractions 21 and 22, M- size exclusion chromatography fraction 33.

In order to determine the composition of BceAB type ABC transporter purified in 0.005% MNG3, sedimentation-velocity analyses using analytical ultracentrifugation (AUC) were performed. The detergent concentration was chosen because it is only five times higher than the critical micelle concentration (cmc) of the detergent and at higher concentrations we experienced interference in the readings by the micelles. The experiments were done at various BceAB transporter concentrations and analyzed in terms of a distribution of sedimentation coefficients $c(s)$, allowing a qualitative evaluation of protein homogeneity and self-association capacity. From the experimental s-values, we derived corrected s_{20w} values, corresponding to pure water density and viscosity at 20 °C. BceAB transporter purified at 0.2 mg/ml in 0.005% MNG3 by size exclusion chromatography was used without dilution, and diluted at 0.1 mg/ml and 0.04 mg/ml in the elution buffer. The experimental sedimentation velocity profiles obtained for the three protein concentrations could be nicely fitted according

to the *c*(*s*) analysis, as shown in Figure 2.11 A and B. In addition to a contribution at 2.6 S (s_{20w}= 4.1 S), which represents almost certainly MNG3 micelles, different complexes are detected, with approximately the same proportions for the three protein concentrations (Figure 2.11. C). The largest peak accounting for about 60% of the signal, has a mean sedimentation coefficient value of *s*=6.55 S (s_{20w}= 10.3 S).



D

BceB:BceA	MW_{prot} (kDa)	f/f_{min}= 1.25 B_{MNG3} (g/g)	f/f_{min}= 1.25 MW_{MNG3} (kDa)	f/f_{min}= 1.25 $MW_{complex}$ (kDa)	f/f_{min}= 1.4 B_{MNG3} (g/g)	f/f_{min}= 1.4 MW_{MNG3} (kDa)	f/f_{min}= 1.4 $MW_{complex}$ (kDa)
1:1	102	2,1	214	316	2,7	275	377
1:2	130	1,3	169	299	1,8	234	364
2:1	176	0,65	114	290	1	176	352
2:2	204	0,4	82	286	0,65	133	337
2:4	260	0	0	260	0,2	52	312

Figure 2.11. Sedimentation velocity of BceAB type ABC transporter in presence of 0.005% MNG3. A) Superimposed experimental sedimentation profiles obtained with interference optics in 3mm optical patch-length center-piece, and the fitted curves from the *c*(*s*) analysis using Sedfit, of BceAB type ABC transporter at 0.2 mg/ml in 100 mM Tris pH 8, 150 mM NaCl, 0.005% MNG3. Reference buffer is without detergent. The last profile corresponds to 6 h sedimentation at 42 000 g and 4°C. The statistical noise has been subtracted. B) Superimposed differences between the experimental and fitted curves from sedimentation profiles above. C) *c*(*s*) analysis of BceAB, at 0.04, 0.1 and 0.2 mg/ml (green, blue, and purple lines respectively). D) Analysis of the *s*-value of the main complex (*s*=6.55 S) in terms of bound MNG3, considering different association states, for a globular protein complex with f/f_{min}=1.25 and an irregular shape with f/f_{min} =1.4, respectively. In red are indicated more plausible hypothesis.

The value of s is related to the protein-detergent complex composition, thus the BceB:BceA stoichiometry and the amount of bound MNG3, and shape. The Svedberg equation was used in the form [66]:

$$s = (MW_{prot}(1 - \rho\,\bar{v}_{prot}) + MW_{MNG3}(1 - \rho\,\bar{v}_{MNG3}))/(6\,\pi\,N_A\,\eta\,f/f_{min}\,R_{min})$$

The amount of bound detergent is reported as B_{MNG3} in g/g protein or molecular weight, MW_{MNG3}. The shape is expressed by the frictional ratio f/f_{min}. The parameters required for the calculation are the molar mass of BceB and BceA polypeptide chains, 75 and 28 kDa respectively; the partial specific volumes $\bar{v}$ of the different components, 0.76, 0.743, and 0.796 mg/ml for BceB, BceA and MNG3 respectively, and the solvent density and viscosity, ρ=1.007 g/ml and η=1.567 cp respectively. N_A is Avogadro's number; MW_{prot} and $\bar{v}_{prot}$ are calculated from BceB:BceA stoichiometry; R_{min} is the minimum radius and is calculated from MW_{prot}, MW_{MNG3}, $\bar{v}_{prot}$ and $\bar{v}_{MNG3}$ [66].

In Figure 2.11 D, we hypothesized different BceB:BceA stoichiometry and two frictional ratios to derive the amount of bound MNG3 from the measured s=6.55 S (s_{20w}= 10.3 S) value. We considered one or two BceB subunits associated to one to four BceA subunits. The amount of bound detergent was between 0 and 2.7 g/g, or 0 and 275 kDa, and the total mass of the protein-detergent complexes was between 260 and 380 kDa. Nonetheless, all of the latter values were consistent with the apparent molecular mass of the complex from gel filtration data (2.10. A) of approximately 310 kDa.

Previous studies of ABC transporters, specifically BmrA [196], BmrC/D and PatA/PatB [24, 80, 196] solubilized in DDM using AUC described them with $s_{20w}\approx$ 8.3 S corresponding to a dimer with a frictional coefficient f/f_{min} of 1.4. PatA monomer in DDM sedimented at s_{20w}= 5.6 S with f/f_{min}= 1.3. f/f_{min} is commonly between 1.2 and 1.3 for globular species and a rather high f/f_{min} of 1.4 indicates an irregularly shaped protein. The amount of bound detergent was about one gram of DDM per gram of dimeric protein. BmrA solubilized in MNG3 sediments at s_{20w}= 8.2 S corresponding to B_{MNG3}= 0.45-0.7 g/g for f/f_{min}= 1.25-1.4 (Christine Ebel, IBS, unpublished data).

When applying these findings to the studied BceAB type ABC transporter, we made several observations. First, the amount of bound detergent expressed in g/g should be considered with caution since the BceA subunit might or might not bind detergent. Second, the previously characterized ABC transporters have six transmembrane helices for a monomer, twelve for a dimer, while the studied BceAB type ABC transporter has ten

predicted transmembrane helices for the monomer, which should affect the amount of bound detergent. A value B_{MNG3} of 0.7 – 1.2 gram per gram of transmembrane protein should correspond to MW_{MNG3} of 50 – 90 kDa for a complex comprising one BceB and 100 – 180 kDa for a BceB dimer. Third, since the BceAB transporter has a large extracellular loop, which could cause some shape irregularities, therefore it is more likely to consider a frictional ratio of 1.4.

The hypothesized complexes with BceB:BceA 1:1 and 1:2 stoichiometry led to an amount of bound detergent of more than 170 kDa, which is unrealistic as described above. The two scenarios highlighted in red: with BceB:BceA 2:1 and 2:2, were acceptable since the amount of bound detergent is between 130 and 180 kDa with f/f_{min} of 1.4. The last scenario with BceB:BceA 2:4 was rejected since no detergent would be bound.

The non-rejected 2:1 stoichiometry by AUC seems very unlikely to us. Indeed ABC transporters need two NBDs and it does not appear probable to have a single NBD bound to a dimer of TMDs and a second, identical NBD free in a solution. In conclusion, the AUC results suggested that the BceAB type ABC transporter exists in the BceB:BceA ratio of 2:2. Interestingly, the ratio of the homologous BceAB transporter from *B. subtilis* was established as BceB:BceA 1:2 [59], which does not correspond to our findings in the pneumococcus. Unfortunately, the stoichiometries of homologous complexes in other organisms are unknown to make effective comparaisons.

To further confirm our conclusions, native mass spectrometry should be performed directly on the membrane containing the overexpressed protein.

2.3 Specific ATPase activity studies of BceAB complex in detergent

Activity testing of a protein is a legitimate quality control test, which ensures that the protein of interest has been folded correctly. It is also an excellent way to test the most promising detergent in order to avoid as much denaturation of the protein as possible. In this activity assay, we took advantage of the end of glycolysis reaction, which allows ATP regeneration through NADH oxidation followed real-time by a decrease of absorbance of 340 nm as described by Jault *et al.* in 1991 [112].

We first pre-heated the assay buffer with 4 mM ATP at 37 °C for five minutes and then mixed with a certain amount of protein. We then measured the activity for ten minutes at 37 °C (Figure 2.12. A). As a negative control, we mixed the protein in the assay without ATP to ensure NADH is not oxidized by any contaminant. Another control for ensuring the assay quality was in absence of the protein. We injected ADP into the assay at 2.5 minutes and we

observed a steep decrease in absorbance as ADP was converted back to ATP. A further control was the addition of 5 mM Ortho-vanadate, a known ABC transporter inhibitor. We observed that vanadate practically stopped the protein activity, suggesting that it is not a contaminant, but the protein itself which drives the activity as it has been observed previously in BmrA or PatA/PatB [24, 220]. We next added different amounts of the BceAB type ABC transporter into the assay and measured the activity, which resulted in 23.6 nmol of ATP/min/mg of protein ± 3.2, nevertheless the ATPase activity varied greatly between different preparations and often reached the activity of ~ 100 nmol/min/mg. Various purified ABC transporters have displayed a high variety of activity ranging from ~ 120 nmol/mim/mg by $MalFGK_2$ from *E. coli* [4], ~ 350 nmol/min/mg by PatA/PatB from *S. pneumoniae* [24] to 1.5 µg/min/ml by BmrA from *B. subtilis* [176]. Therefore the activity of the purified BceAB type protein is found within the previously published range of activity.

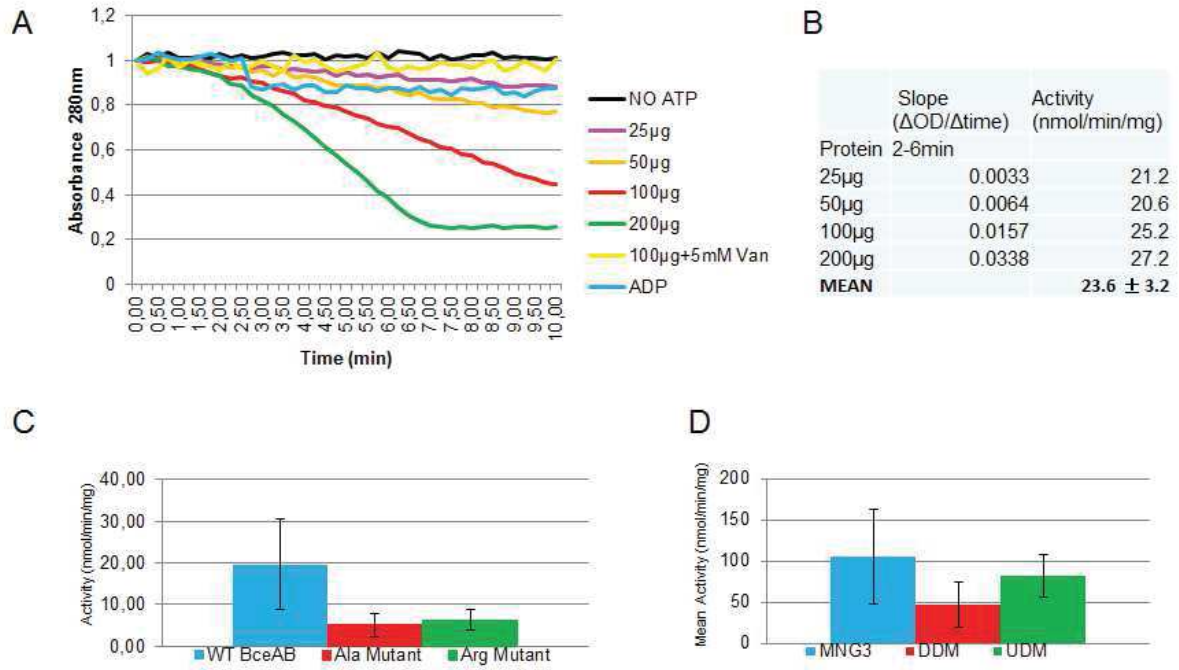


Figure 2.12. ATPase activity experiments of the BceAB type ABC transporter in detergent. The activity assay was performed in 50mM Tris pH8 and 100mM NaCl buffer supplemented with 32 µg/ml lactate dehydrogenase (*Roche*), 60 µg/ml pyruvate kinase (*Roche*), 4 mM phosphoenolpyruvate, and 0.4 mM NADH and 4mM ATP unless indicated otherwise. The assay was incubated for 5 min at 37°C and after the addition of the protein, the reading was performed every 20 seconds for 10 minutes at 37°C. A) Raw curves of ATPase activity assay readings. Black line represents negative control of 100 µg of the ABC transporter in the assay without ATP. Blue line represents another control is the system without the protein where we added ADP at 2.5 minutes and we see a drop in absorbance as the system recharges the ADP to ATP and uses NADH. Pink, yellow, red, and green lines show activity of 25, 50, 100, and 200 µg/ml of protein respectively. Yellow shows activity of 100 µg of the BceAB type ABC transporter in presence of 5mM Vanadate. B) Calculation of specific activity in nmol/min/mg of protein using the slope of activity between 2 and 6 minutes of the experimental run. This is a representative result of different protein preparations. C) Average ATPase activity of WT BceAB type transporter and alanine or arginine mutants. By point mutation, alanine or arginine replaced the lysine in the Walker A region involved in ATP binding. D) Test of purification in different detergents following overnight solubilization in MNG3.

We also tested the activity of the protein in different detergents. After overnight solubilization in 1% MNG3 we followed the normal purification protocol up to the column wash, where we washed the Ni-NTA column and purified the BceAB type ABC transporter in the normal buffer containing either 0.01% MNG3, 0.05% *n*-Dodecyl β-D-maltoside (DDM), 0.15% *n*-undecyl-β-D-maltopyranoside (UDM), and 2.65% octyl glucoside (OG). Purification in OG caused the protein to precipitate within five minutes after elution. The concentration of the protein purifications in MNG3, DDM, and UDM were 1.6 mg/ml, 1.6 mg/ml, and 2.0 mg/ml respectively. The activity test was then performed at several protein concentrations (25, 50 and 100 µg/ml) and in the respective detergent in the assay medium. Since the MNG3 generally showed the highest protein activity we decided to continue using this detergent.

This was a rather expected result, since compared to DDM or UDM, MNG3 harbors a central quaternary carbon , which restrains conformational flexibility causing the MNG3 detergent to be “milder” to the membrane protein [30]. Similarly, as stated above, BmrA was more active and more stable when solubilized in MNG3 than in other detergents such as DDM [249].

We next created mutants of the BceAB type ABC transporter where we replaced the lysine in the BceA (NBD) Walker A region by alanine (K48A) or arginine (K48R). These mutations, as a result, should prevent the correct positioning and hydrolysis of ATP in the ATP binding pocket and thus inhibit protein activity as seen in various ABC transporters: BmrA [178], PatA/PatB [24], and BmrCD [80]. The point mutations did not have an impact on the protein expression or on the purification. As seen in Figure 2.12. C, the activity of the WT was on average four times higher than that of the mutants. We therefore confirmed the hypothesis from the vanadate experiment that the majority of the activity observed is due to the purified BceAB type ABC transporter and not to a contaminant in the purification.

We also tested the protein activity in presence of various amounts of bacitracin, but no difference in activity has been observed (Figure 2.13.). This result may appear unexpected in comparison to LmrCD, a MDR ABC transporter in *L. lactis* , whose activity increased two times in presence of its substrate rhodamine [209]. Human P-glycoprotein, another MDR ABC transporter, was shown to have its ATPase activity stimulated several-fold by some of its substrates [213]. On the other hand, a homologous protein, BmrA of *B. subtilis*, was stimulated by only 25% with the addition of a substrate, Hoechst 33342, to the assay [220]. Similarly, another homologue from *L. lactis*, LmrA [107], comparable results to ours have been observed, where the addition of several transported substrates to the assay containing the purified protein did not increase the ATPase activity of the BceAB type ABC transporter.

In context of the result seen in the qPCR experiments, the BceAB type ABC transporter is likely expressed at low levels and continually hydrolyzes ATP. Even though ATPase activity does not show the efficiency of transport activity [51] we may yet conclude, that upon challenge of the pneumococcus by bacitracin, and presumably other peptides, the BceAB type ABC transporter is then overexpressed high levels to confer resistance of the bacteria towards the AMP.

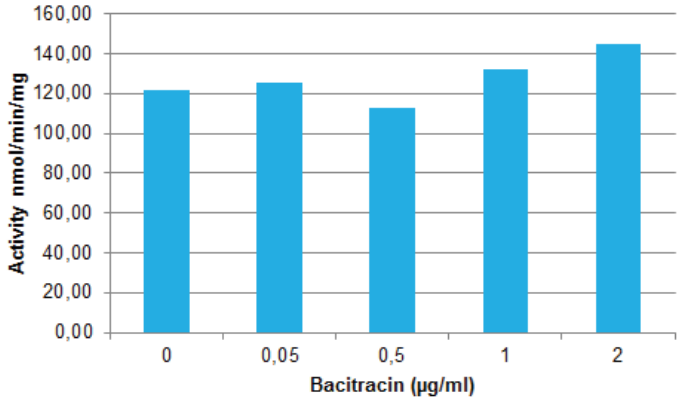


Figure 2.13. ATPase activity of BceAB type ABC transporter in the presence of bacitracin. The assay was in 50mM Tris pH8 and 100mM NaCl buffer supplemented with 32 µg/ml lactate dehydrogenase (*Roche*), 60 µg/ml pyruvate kinase (*Roche*), 4 mM PEP, and 0.4 mM NADH and 4mM ATP and 50µg of purified BceAB type ABC transporter on a Ni-NTA column. The graph is a representative result of three different ATPase activity experiments of different protein preparations.

2.4. Insertion of functional BceB type TMD into Nanodiscs *(In collaboration with Yann Huon de Kermadec, IBS)*

In order to better simulate the behavior of the BceAB type ABC transporter in its native state, we reconstituted the BceAB transporter in nanodiscs. After the protein transfer into the lipid bilayer, we removed the detergent by treating the solution with biobeads. Unfortunately we did not succeed to purify the BceB containing nanodiscs form empty ones on a Ni-NTA affinity column probably because the histidine tag could have been buried in the nanodiscs or otherwise masked. We then purified the sample by size exclusion chromatography (Figure 2.14. A). The fractions of the main peak (~ 310 kDa) lanes 19-22 displayed significant BceB presence in the nanodiscs (Figure 2.14. B and C). Considerable amount of MSP may be observed as well suggesting there were many empty nanodiscs present in the sample. However, no band for BceA was observed neither in the Coomassie blue nor in the Western blot, suggesting that only BceB has been inserted into the nanodiscs and the BceA has dissociated during the process. To estimate the amount of BceB in the sample, we made a Coomassie blue stained gel with known amounts of protein in detergent. When plotted on a graph we could calculate the estimated BceB concentration to 0.22 mg/ml ± 0.06.

We then proceeded to test the ATPase activity of the protein in nanodiscs. We repeated the experiment with three different protein preparations. For lone BceA we used the BceA obtained during gel filtration as seen in lane M in Figure 2.10. B and C. To test the

complex in nanodiscs, we mixed the BceB in nanodisc together with the BceA obtained as described above in 1:1 w/w ratio. The protein complex purified from the same size exclusion chromatography was used as the BceAB type ABC transporter in detergent. While individual components display some ATPase activity, similar to those seen to the complex in detergent, the mixture of BceB and BceA shows a great increase in ATPase activity. In comparison, the activity of the BceAB transporter in nanodiscs is almost 5 times higher than in detergent. Also interestingly, the activity of individual subunits is similar to the one in detergent suggesting a high level of dissociation of the BceAB complex in detergent solution. Yet, due to the increased activity in nanodiscs, the dissociation of the complex seems reversible. Correspondingly to our results, Galian *et al.* in 2011 [80] observed that the heterodimeric ABC transporter BmrCD showed a quite high level of dissociation during solubilization and purification in detergent. When a single subunit of the BmrCD transporter was tagged, the predominant species purified was a monomer of either BmrC or BmrD. Nevertheless, the dissociation was reversible when the detergent concentration was lowered or the proteins were reconstituted in proteoliposomes. They also observed an eight time increase of ATPase activity during reconstitution in proteoliposomes better mimicking the membrane than detergents.

Moreover, when the lipid A ABC transporter from *E. coli*, MsbA, was reconstituted in proteoliposomes, the initial experiments showed a significant decrease in ATPase activity compared to activity in DDM [67]. Nevertheless, when MsbA was reconstituted in nanodiscs, the protein activity increased five to ten times to ~ 200 nmol/min/mg compared to the activity in DDM [122].

We also tested the activity of the protein in presence of bacitracin, but no difference in activity has been observed as when the protein was in detergent (data not shown). We may then assume that the substrate binds to the loop as there is no detergent present to inhibit substrate binding. Therefore, as mentioned before, the resistance mechanism is most likely driven by high expression of the BceAB type ABC transporter rather than increasing the transporter activity. Again, it has been observed before with BmrCD [80] and BmrA [220] [176], that while the ABC transporter transport different substrates in vesicles, only a few of these substrates stimulated the ATPase activity upon reconstitution of BmrA in proteoliposomes. This is due to the fact that different drugs may interact differently with the same ABC transporter by binding to different sites or by limiting the transition state of the protein [51, 80, 244].

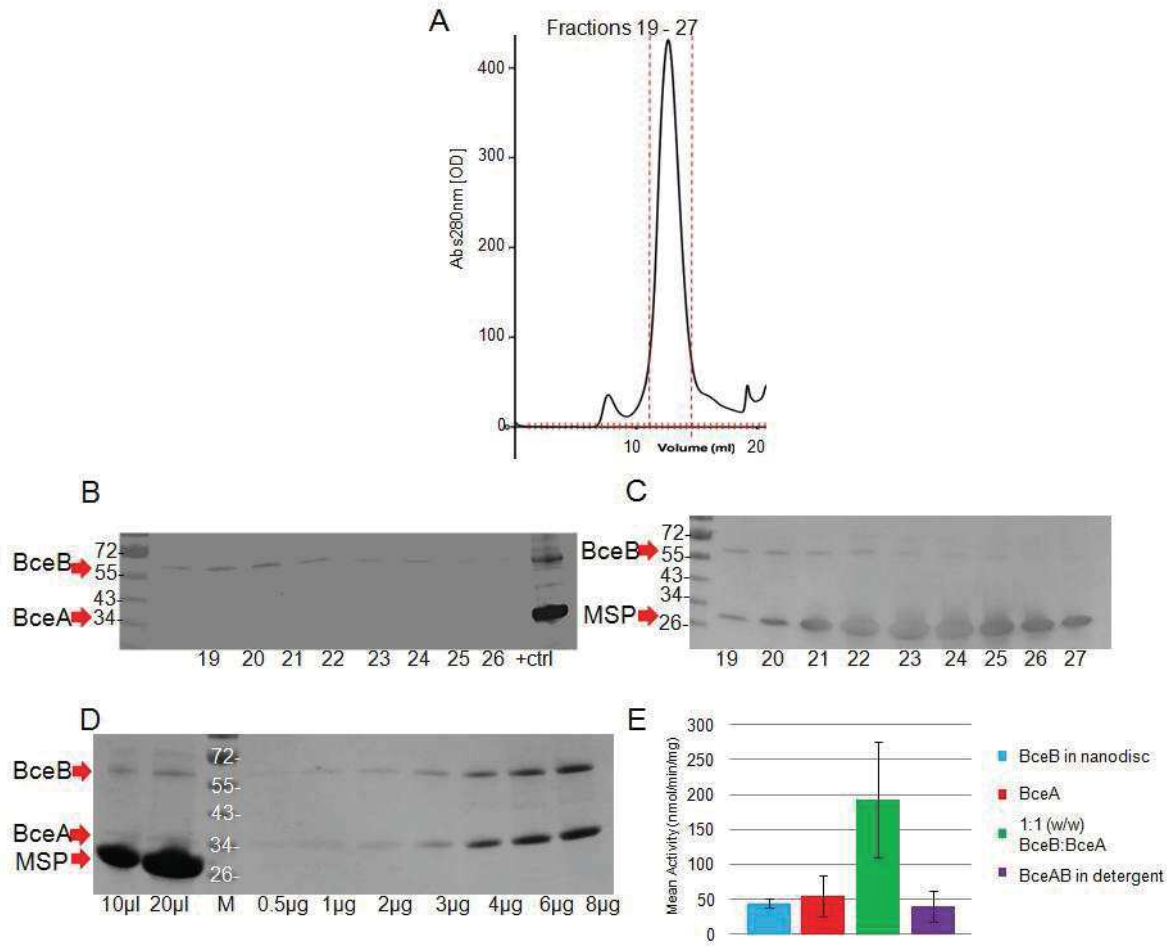


Figure 2.14. Insertion of BceB into nandisk and measurement of its ATPase activity. A) Size exclusion chromatogram of BceB insertion into nanodisc. The BceAB transporter was previously purified on Ni-NTA column. B) and C) SDS-PAGE gels (Coomassie blue stain and WB respectively) of the chosen fractions from the chromatogram. 7.5 µl of total 0.5 ml fraction were loaded onto the gel. D) Coomassie blue gel used for calculation of BceB concentration in the nanodiscs. E) ATPase activity of BceB in nanodisc alone, the BceA alone, the mixture of BceB in nanodisc and BceA in a one to one ratio (w/w), and BceAB transporter activity after size exclusion chromatography.

Summary and perspectives

In the beginning of this PhD project, the BceAB-RS type ABC-TCS complex were poorly described. Only theoretical data has been known in regards to the stoichiometry of the complex and the functioning mechanism.

As stated above, this is the first study to describe the complex on the molecular and cellular level in the *S. pneumoniae*. Our *in vivo* experiments provide important insights into the functioning of the complex. As it has been observed in *B. subtilis* [218], both the ABC and TCS components are necessary in order for the complex to function properly. A loss of function mutation in one component may have serious consequences for the pneumococcus in terms of loss of resistance to AMPs. Since many of the bacteria occupying the same niche as the pneumococcus produce a variety of AMPs it might have fatal consequences for the bacterium. Additionally, we may hypothesize that the BceAB-RS type system in the pneumococcus only transports bacterial AMPs based on the growth curve and infection experiments.

It is also the first study to show that the BceRS drives the overexpression of the BceAB in *S. pneumoniae*. It is, in fact, by the overexpression of the BceAB that the bacteria are able to resist certain antimicrobials, as the presence of bacitracin did not increase the protein activity during *in vitro* experiments and *in vivo* experiments revealed a great increase of expression of the transporter. It would be interesting to delete the large extracellular loop of the BceAB transporter and observe if the signaling is lost upon AMP stress, as it has been shown in *B. subtilis* [13].

Last year, Dintner *et al.* [59] purified the BceAB type ABC transporter from *B. subtilis* and by size exclusion chromatography, they determined the stoichiometry to be one BceB subunit to two BceA subunits, but without protein activity, the data is open to doubt. We purified a functional protein and combining AUC data with size exclusion chromatography we determined the stoichiometry to be two BceBs to two BceAs.

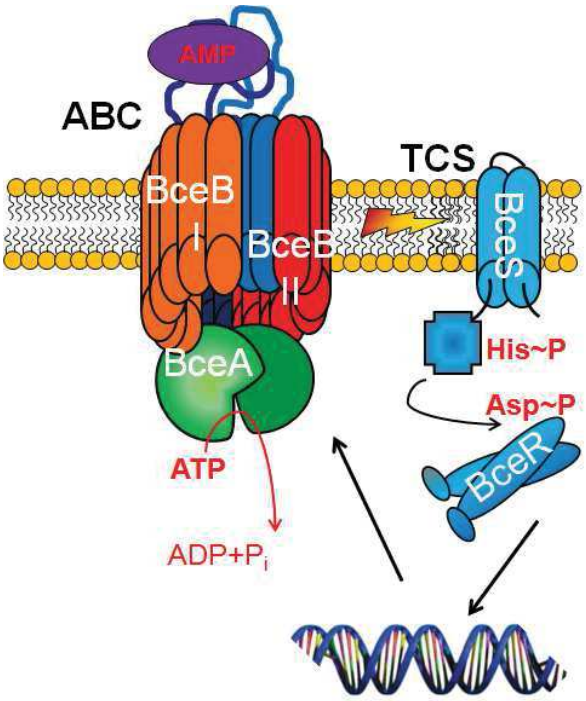


Figure 2.15. New BceAB-RS type ABC-TCS system hypothetical model. A dimer of BceB senses the AMP presence and communicates to the BceS to overexpress the BceAB transporter through the soluble BceR. A dimer of BceRs hydrolyze ATP and transport the substrate.

We are now able to purify each subunit of the complex and study and especially a functional BceAB type ABC transporter. Due to the nature of its function, which is resistance, it is important to continue studying its functional mechanism and structure. For further mechanism studies, an option would be using NMR of inside-out vesicles containing the protein and observing the transport of substrates into the vesicle. This kind of experiment would provide the possibility to observe if the BceAB transporter is an importer or exporter as it has been shown in studies of other transporters by Krumpochova *et al.* in 2012 [132]. Even though based on informatics analysis the protein is an exporter, energetically speaking, it would cost less ATP to import the AMP for internal digestion.

Structural studies could give other important insights in respect of the function. To begin with, it could be an option to have a rough 3D structure of the protein using the cryo-EM technique of the BceB in nanodisc. This technique has been successfully used in studying membrane proteins by Frauenfeld *et al.* 2011 and Gogol *et al.* 2013 [77, 88].

Additional structural insights of the 3D structure might be provided by small angle X-ray scattering (SAXS) or small angle neutron scattering (SANS). It has been demonstrated in the past [26, 133, 206], that it is possible to use these techniques for membrane proteins in

either detergents or nanodiscs to have a rough 3D structure and therefore form more precise hypotheses of the functioning mechanism. Furthermore, the analysis of these structures gives important clues to facilitate eventual crystallography experiments, which would solve the structure at the atomic level. The fact that we observe a higher activity in nanodisc than in detergent also suggests that the protein might be in a more natural conformation. The transfer of a membrane protein from nanodisc to lipidic-cubic phase has been successfully done by Gordeliy's team at the IBS when crystallizing bacterial rhodopsin (publication submitted). This might be a good approach for the BceAB type ABC transporter as well for crystallography experiments.

Another remarkable subject to study is the interaction of the BceAB transporter with the BceS. Theoretically, according to Dintner *et al*. in 2014 [59] the proteins interact by the transmembrane domains. Since we encountered the problems with the streptavidin tag, such as low binding of the streptavidin tagged protein to the column probably given by delicacy of the Strep-Tactin® column (*IBA*) in presence of MNG3, maybe it could be a good solution to tag the protein with histidine on either end, as we attempted, but with a possibility to cut the tag. Afterwards it could be possible to attempt a pull down experiment on a nickel affinity column and observe if the BceS elutes with the BceAB.

Alternatively, as shown by Winkler and his team in 2013 [134, 212], it should be feasible to tag the BceB or the BceS with a FLAG tag directly in the pneumococcus. After growing several liters of culture with tagged bacteria and wild-type bacteria and lysing the cells, the samples might be passed through a column containing α-FLAG beads. Using silver staining it then may be possible to identify additional bands on the gel after passing the sample through the column.

Yet another alternative for observing BceAB-BceS co-localization would be using microscopy techniques such as PALM or STORM [79, 215, 224]. Again, it would involve tagging the proteins directly in the *S. pneumoniae*, but with a fluorescent tag such as GFP or YFP. Following single molecules then may allow us to follow individual proteins and their co-localization.

Part 3: Materials and Methods

1. Experiments on the *S. pneumoniae* D39

1.1. Bacteria and growth conditions

The *S. pneumoniae* used in these studies was the virulent type 2 D39 strain. Pneumococci were routinely grown at 37°C with 5% CO_2 in air in Todd-Hewitt broth (*Difco*) with 0.5% w/v yeast extract when necessary (THY) or on Columbia agar (*Difco*) plates containing 5% v/v of horse blood. When appropriate, antibiotics were added to media with the following concentrations: chloramphenicol (Cm): 4.5–10 µg/ml and/or kanamycine (Kan): 50 µg/ml.

1.2. DNA extraction

The National Centre for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/Blast) was used for DNA and protein BLAST searches.

Genomic DNA was isolated from a 5 ml culture of *S. pneumoniae* D39 grown to optical density (OD) 0.3. The culture was spun down at 20 000 g for 10 minutes and the pellet resuspended in 500 µL PBS containing lysozyme (10 µg/ml) and mutanolysine (0.5 µg/ml) and incubated at 37°C for one hour. Then the genomic DNA was extracted using the High Pure PCR Template Isolation kit (*Roche*) according to the manufacturer's instructions. The quality and quantity of the DNA extraction was performed using NanoVue™ Spectrophotometer (*GE Healthcare*)

1.3. Construction of deletion mutant strains

For in-frame deletion of the ABC genes (*Spd0804* and *Spd0805*), a construct was created in which 848 bp of flanking DNA 5′ to the *Spd0804* ATG (primer F1 Table 1.1.) and 798 bp of flanking DNA 3′ to the *Spd0805* open-reading frame (ORF) (primer F6) were amplified by PCR from *S. pneumoniae* D39 genomic DNA and fused to the chloramphenicol resistance marker (*CAT*, amplified from pR326 [38], with primers F3 and F4) by overlap extension PCR [142] as shown in the results section. The fragments were amplified using 2.5 µl of primers (100 µM), 1 µl of 10 mM dNTP (*Thermo Scientific*), 10 µl of 5x High Fidelity buffer (*Thermo Scientific*) and 0.5 µl of Phusion polymerase protein (*Thermo Scientific*) in a 50 µl final volume. The PCR program was as follows: 98 °C for 30 seconds, then a 35 time repeat of 98 °C for 10 seconds, 48 °C for 30 seconds, and 72 °C for 90 seconds, after the last cycle at 72 °C for 8 minutes and samples were kept at 4 °C. The DNA samples were analyzed between each step by electrophoresis in 1 % agarose gel and TAE buffer (Tris pH8, acetic

acid and EDTA). The fragments were purified using the QIAquick Gel Extraction Kit (Qiagen) and eluted in 25 µl of water and concentration measured using NanoDrop. Equal weight of the three fragments were mixed and submitted to the same PCR program to be fused together. Primers used for the overlap extension PCRs were F1 and F6. We purified the fragments from the agarose gel using the same kit as previously.

S. pneumoniae genome was afterwards transformed by homologous recombination and allelic replacement using competence stimulating peptide (CSP-1) and standard protocols [98, 136] where 15 ml of D39 was grown to OD_{600nm} 0.03 and the culture was separated into 9 and 3 ml and spun down for 10 minutes at 20 000 g. The culture was then resuspended in 1 ml THY pH8 containing 0.2% (v/v) BSA and 1mM of $CaCl_2$. Then 5 µg/ml of CSP1 was added for 7 minutes incubation at 37 °C. After addition of of 20 µl of purified PCR product growth was carried out for 5 hours. Finally, 100 µl of bacteria were spread on Columbia blood agar plates with chloramphenicol in serial dilutions from pure bacteria to 10^{-6}. Only the 10^{-6} dilution was spread for the control culture, which underwent the same treatment as transformed bacteria without any DNA being added.

For deletion of the TCS genes (*Spd1445* and *Spd1446*), a construct was created as described above: 850 bp of flanking DNA 5′ to the *Spd1445* ATG (primer H1) and 757 bp of flanking DNA 3′ to the *Spd1446* ORF (primer H6) were amplified by PCR from *S. pneumoniae* D39 genomic DNA and fused with the kanamicine resistance marker (*KANA*, amplified from pLIM100, courtesy of Laure Bellard, with primers H3 and H4). The final product was 2610 base pairs long. PCR, purification and transformation protocols were identical to the ones described above.

The mutations were tested by PCR on positive clones amplifying the insert by outside test primers T1 and T2 for ABC deletion and T3 and T4 for TCS deletion. The expected sizes for the strains were WT 4804 bp ΔABC 3155 bp and WT 4628 bp ΔTCS 3900 bp respectively. The constructs were then additionally verified by DNA sequencing by Beckman Coulter Genomics, Cogenics Online.

Fragment name	Primer position	Primer sequence
F1 - F	-848	GCTGTAATTTAGTCGGCAATG
F2 - R	-24	TCAAACAAATTTTCATCAAGCTTGA ATCTCCTTTCTTAATATCCC
F3 CAT - F	-14	GGGATATTAAGAAAGGAGATTCAAG CTTGATGAAAATTTGTTTGA
F4 CAT - R	+1963	CATTTGGACAATCTTACGATAACTCT AGAACTAGTGGATCCCCCGG
F5 - F	+1963	CCGGGGGATCCACTAGTTCTAGAGT TATCGTAAGATTGTCCAAATG
F6 - R	+2768	GCTTGGACTACAAGTCACC
T1 - ABC - F	-217	CACGTAAACGCAAAGAAGC
T2 - ABC - R	+5006	CACAAGATTCTTTCCATCAC
H1 - F	-397	GGAATTTCCAGCATCATACC
H2 - R	-56	CATTAAAAATCAAACGGATCCCATG CTAGATGGTCTGAAAC
H3 KANA - F	-56	GTTTCAGACCATCTAGCATGGGATC CGTTTGATTTTTAATG
H4 KANA - R	+931	CACTGTCGTTCCTTTTCCGCGTCTAG AAAGACTGAG
H5 - F	+931	CTCAGTCTTTCTAGACGCGGAAAAG GAACGACAGTG
H6 - R	+1732	CTTCAACTTGACTGACTACC
T3 - TCS - F	-26	CTGTTCGTGAATTCGAATCTG
T4 - TCS - R	+4656	CGAAGTAGAGCTGAAGTTC

Table 2.1. Primers used for creating *S. pneumoniae* D39 deletion mutants. Position of the primer is given upstream (-) or downstream (+) from the first ATG of the NBD and HK.

1.4. Growth of *S. pneumoniae* wild-type and mutant strains and determination of drug sensitivity

Growth of *S. pneumoniae* strains was determined using a protocol previously described [24]. THY broth was inoculated with 1/100 *S. pneumonie* cultures grown to a logarithmic phase. Cultures (300 µl) were grown in 96 well plates (*Falcon* flat-bottom MICTOTEST^{TM}) sealed with a transparent film (Duck-tape). Plates were incubated at 37 °C in a FluoStar Optima (*BMG Labtech*). Growth was monitored by recording the turbidity at OD_{600nm} every 20 min for 15 hour and after a 7 second orbital shaking (270 rpm). Drug

sensitivity was assessed by adding various concentrations of different drugs in the culture wells as mentioned elsewhere in the results section of this work. Generation time in each condition was calculated from linear phase of the semi log plots corresponding to the log-to-log transition part of the curve using Excel. Generation per hour was calculated with the formula 1/(ln(2)/*m*) where m is the slope of the linear phase.

1.5. Capsule size measurement

Capsule size of the *S. pneumoniae* was verified using the following protocol. *S. Pneumoniae* WT and mutant strains were routinely grown in 13 ml of THY to the OD_{600nm}= 0.5. The cultures were then centrifuged at 20 000 g for ten minutes. The pellet was resuspended with 100 µl PBS, transferred into Eppendorph tubes and centrifuged. All of the supernatant was carefully removed and the pellet weighed. The volume of final resuspention volume was then calculated accordingly: (OD_{600nm} x 266 µl) - weight = x µl PBS resuspention buffer. 35 µl of the resuspended bacteria were loaded on LightCycler® Capillaries (*Roche*) and the capillaries were centrifuged at 6 000 g for 7 minutes.

1.6. Experimental design for Drosophilae infection experiments

S. Pneumoniae WT and mutant strains were concentrated from a 200 ml culture in the mid-higher part of the linear phase (OD_{600mn}= 0.3–0.6). Bacteria were spun for 10 minutes at 20 000 g and then resuspended in 500 µl of THY. The OD_{600nm} was measured of a 10 times dilution. For OD_{600nm} between 0 and 1, there is a direct relation of the number of bacterial cells present in the suspension where OD_{600nm} = 0.1 = $1x10^8$ colony forming units (CFU) per ml. According to the measured OD_{600nm}, the sample was diluted to $2x10^{10}$ CFU/ml including 20% glycerol. The bacterial aliquots were stored at -80 °C. CFU was calculated from a serial dilution of bacteria in sterile PBS. The concentrations plated overnight on Columbia blood agar were $10^{-7,}$ 10^{-8} and 10^{-9} dilutions.

Flies were raised on standard culture medium at 25 °C. Briefly, for infection, fifty 3–5 days old males were jabbed in the thorax with a thin needle that had been previously dipped into 50 µl of the concentrated culture of *S. pneumoniae* described above. Then the flies were placed into vials by ten and placed for 18 hours at 25 °C. Next, the flies were placed into a 30 °C incubator and then counted at intervals to measure survival.

1.7. qPCR

S. pneumoniae WT and mutant strains were routinely grown to OD_{600nm}=0.3. Then 1 µg/ml of bacitracin was introduced to the growth media for 30 minutes. Samples were taken at time points shown in the results section. Next, the cultures were mixed with RNA protect (*Qiagen*) at 1:1 ratio and incubated at room temperature for 5 minutes and centrifuged for 10 minutes at 5 000 g at 4 °C. The supernatant was decanted and the pellet was then stored at -80 °C. The bacteria was resuspended in 100 µl of sterile Tris-EDTA (TE), containing 15 mg/ml lysozyme (*Sigma*) then 20 µl of Proteinase K (20 mg/ml) (*Roche*) were added. The sample was vortexed 5 times for 10 seconds at two-minute intervals. Then 350 µl of lysis buffer were added to the sample with 3.5 µl of β-mercaptoethanol and 25 mg of glass beads (*BioRad*). The cells were lysed by continual vortexing for 5 minutes. The mRNA was extracted using the Nucleospin RNA kit (*Macherey-Nagel*). After elution, RNA samples were incubated with 5 µl of DNAse for 20 minutes at room temperature, then heated for 70 °C for 5 minutes. RNA concentration was measured using Nanovue (*GE Healthcare*). For cDNA synthesis 1 µg of RNA was generally used with the SuperScript®III kit (*Invitrogen*) containing a reverse transcriptase, a set of primers, dNTPs, $MgCl_2$ random hexamers and RNaseOUT™ which digests left-over RNA after the cDNA generation according to manufacturer's instructions. Samples were then stored at -20 °C. qPCR measurements were done from 2.5 µl of cDNA mixed with 1 µl (1 µM final) of forward and reverse primers (Figure 3.2.) each and 5 µl iQ™ SYBR® Green Supermix (*BioRad*) and 10 µl of Qsp (*BioRad*) using the CFX Connect™ Optical Module (*BioRad*) program as follows: 95 °C for 3 minutes, then a repeat 40 times of 95 °C for 10 seconds, 55 °C for 30 seconds, and a fluorescence reading.

Primer Name	**Primer Sequence**
NBD - F	CAGGGCAACCAAGTAGAAGC
NBD - R	CGGTGTCAGTTCCATTCAAG
TMD - F	TGCCAGTGAAGCAGAACAAC
TMD - R	CCGATAAAGAAGACACCACCA
HK - F	GGAGAGAGGGAAGCCAAGTC
HK - R	GCAATGGGGGTCTTTATCTG
RR - F	GTTTGGGCGTGATGAGAGTT
RR - R	CGAGCCACATTGACAGAGAG
PatA - F	GCAACCCACATTCACGACTA
PatA - R	CCTGCTACAACCACCTCCAT

Table 1.2. Primers used for qPCR experiments on *S. pneumoniae* D39.

2. Production and Characterization of the ABC transporter and the TCS

2.1. Cloning

Plasmids were prepared by insertion of DNA fragments obtained from *S. pneumoniae* D39 genome (primers in Table 1.3.) into the pRSF-Duet1 or pET-Duet1 vectors (Figure 3.1.). The pRSF-Duet1+ABC plasmid has been prepared in the following manner: a NBD-TMD fragment was amplified from *S. pneumoniae* D39 genome using ABC-F (short) and ABC-R (short) primers. The fragment was amplified using 5 µl of primers (100 µM), 4 µl of 10 mM dNTP (*Thermo Scientific*), 20 µl of 10x pfu PCR buffer (*Promega*) and 4 µl of pfu polymerase protein (*Promega*) to a total volume of 200 µl. The PCR program was as follows: 94 °C for 1 minute, then a 25 time repeat of 94 °C for 45 seconds, 53 °C for 1 minute, and 72°C for 2 minutes, after the last cycle at 72 °C for 10 minutes and samples were afterwards kept at 4 °C. The fragments were subsequently purified in PCR purification kit (Qiagen) and eluted in 30 µl of water. A second PCR was performed using the ABC-F and ABC-R primers that enabled the insertion of a histidine tag on the *N*-terminus of the NBD and the *C*-terminus of the TMD respectively, using the first PCR fragment as a template. Identical PCR program and PCR purification protocol were used. The pRSF-Duet1 plasmid and the second his-NBD-TMD-his fragment were both sequentially digested by first NcoI restriction enzyme (*New-England Biolabs*) and by EcoRI restriction enzyme (*New-England Biolabs*) according to the manufacturer's instructions. After that, the fragment and the plasmid were ligated using Rapid DNA ligation kit (*Roche*) according to manufacturer's instructions. The ligation reaction was used to transform XL10Gold competent bacteria (*Invitrogen*). Positive clones were selected by restriction digest and verified by sequencing (*Beckman Genomics*). Mutations in the Walker A region of the NBD, alanine (K48A) or arginine (K48R) were performed using the Strategene mutagenesis kit and the sets of primers in Table 1.3.

The pRSF-Duet1+HK plasmid has been prepared in the following manner: an HK fragment was amplified from *S. pneumoniae* D39 genome using HK-F and HK-R primers introducing a streptavidin tag on the *N*-terminus of the HK protein. The fragment was amplified using the same PCR protocol as above except 60 °C was used for annealing temperature. The pRSF-Duet1 plasmid and the HK fragment were sequentially digested by first NdeI restriction enzyme (*New-England labs*) and by XhoI restriction enzyme (*New-England labs*) according to the manufacturer's instructions. The fragment and the plasmid

were afterwards ligated as described above as well as bacterial transformation, selection and genetic verification.

The pET-Duet1+RR plasmid has been prepared in the following manner: an RR fragment was amplified from *S. pneumoniae* D39 genome using RR-F and RR-R primers with a histidine inserted on the *C*-terminus of the RR protein. The fragment was amplified using the same PCR protocol as above except 50 °C was used for annealing temperature. The pET-Duet1 plasmid and the RR fragment were both sequentially digested by first NdeI restriction enzyme (*New-England labs*) and by XhoI restriction enzyme (*New-England labs*) according to the manufacturer's instructions. The fragment and the plasmid were then ligated as described above as well as bacterial transformation, selection and genetic verification.

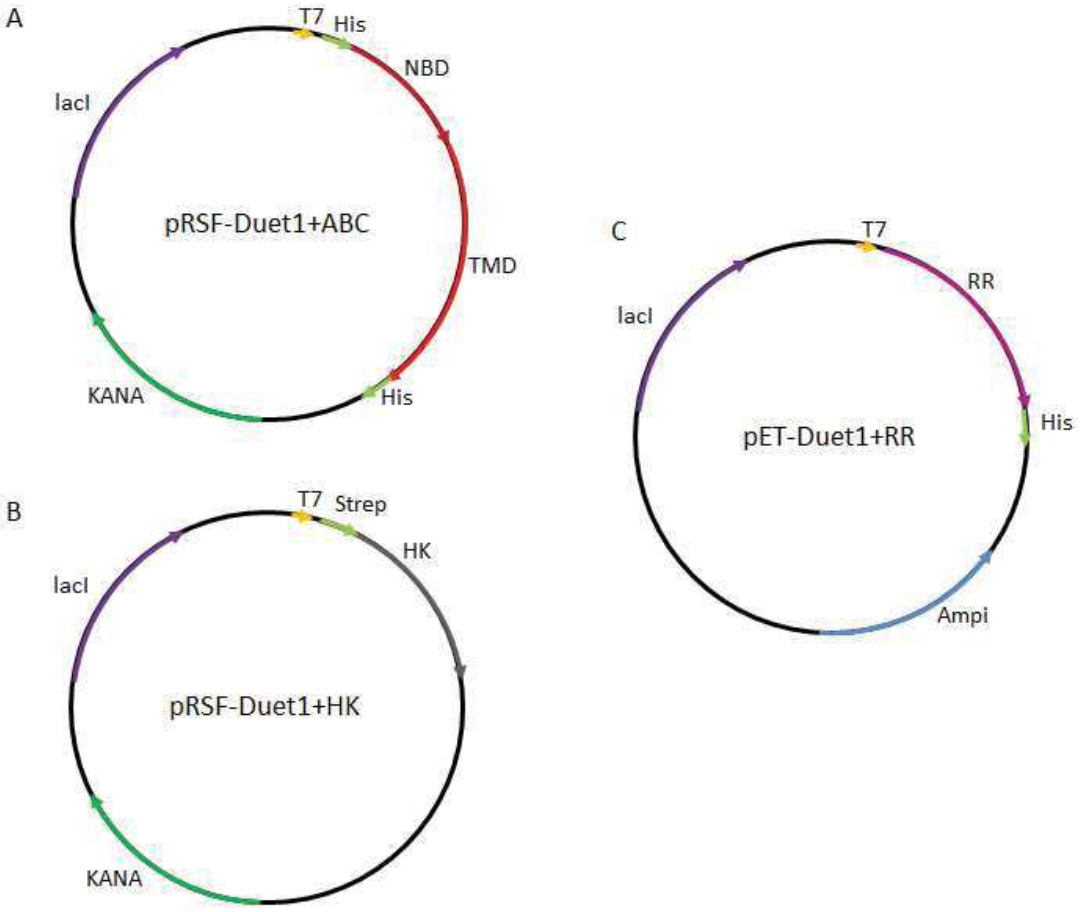


Figure 3.1. Maps of ABC, HK and expression plasmids. pRSF-Duet1 contain a kanamycin resistance cassette, while the pET-Duet1 contain an ampicillin resistance cassette. A) Plasmid pRSF-Duet1+ABC used to express the ABC transporter under the T7 promoter. B) Plasmid pRSF-Duet1+HK used to express the HK under the T7 promoter. C) Plasmid pET-Duet1+RR used to express the RR under the T7 promoter.

Primer name	Sequence
RR - F	ATATAT*CATATG*TCAAGCATGCTTCAATCCG
RR - R	ATATAT*CTCGAG***ATGATGATGATGATGATGATG**TTTTTTCACAAGATTTTATTAATAG
ABC – F (short)	GGACACTTTTAGATGTAAAACAC
ABC – R (short)	CTACATTTGGACAATCTTACG
ABC - F	GATATA*CCATGG*GG**CATCATCATCATCATCATCATCAT**ACACTTTTAGATGTAAAACAC
ABC - R	GAGCTC*GAATTC*CTA**ATGATGATGATGATGATGATGATG**CATTTGGACAATCTTACG
HK - F	GATATA*CATATG***GCTAGCTGGAGCCACCCGCAGTTCGAAAAAA**TGCTTGATTGGAAACAATTTTTTCTAGC
HK - R	ACCAGA*CTCGAG*AATGAGTTCCTGATTCAAGTGAACTC
Point mutation	
Arg - F	CATGGGTGAGTCTGGTTCTGGTCGTTCAACTCTTCTCAATATTCTAG
Arg - R	CTAGAATATTGAGAAGAGTTGAACGACCAGAACCAGACTCACCCATG
Ala - F	CATGGGTGAGTCTGGTTCTGGTGCGTCAACTCTTCTCAATATTCTAG
Ala - R	CTAGAATATTGAGAAGAGTTGACGCACCAGAACCAGACTCACCCATG

Table 1.3. Primers used to clone genes into the plasmids and for point mutation. In italics are shown the restriction sites, tags are marked in bold and annealing sequences are underlined, point mutations are double underlined.

2.2. Bacteria and growth conditions

TOP10, XL10Gold (*Invitrogen*) *E. coli* strains were used for cloning and plasmid amplification according to the manufacturer's instructions. Transformations were performed as recommended by the manufacturer and transformants were selected on LB agar plates containing the appropriate antibiotic.

E. coli BL21(DE3) [162] was employed for over-expression. Bacteria were routinely grown in the Luria Bertani (LB) medium (*AthenaES*) at 37 °C. The transformed bacteria were grown in LB medium with either 50 μg/ml kanamycin or 100 μg/ml ampicillin at 37 °C overnight. 10 ml of the pre-culture were inserted into 1L of LB medium with antibiotic and grown shaking at 180 rpm at 37 °C for 1.5 hours. The temperature was then lowered to 30°C until the bacteria grew to mid-log phase (OD_{600mn} ~ 0.5) when they were induced by addition of IPTG (1 mM final concentration) and left to grow overnight at 20 °C under shaking. Bacteria were collected by low-speed centrifugation (5 000 g during 20 min), washed with PBS (7.5 ml per

liter of culture) and spun again. The cells were then resuspended in a buffer containing 50 mM Tris pH8, 300 mM NaCl and protease inhibitors from a Complete® EDTA Free tablet (1 tablet per 100 ml solution) (*Roche*).

2.3. Protein Expression and Purification

Proteins were purified as described below. All of the steps were either performed at 4 °C or on ice.

2.3.1. RR Sample Preparation

Four liters of bacteria over-expressing the RR protein were spun down at 20 000 g for 30 minutes and resuspended in 40 ml of buffer containing 20mMTris pH8, and 300mM NaCl and a Complete-EDTA free tablet (*Roche*). The cells were afterwards lysed using sonication. The sonication cycle was 2 seconds on and 10 seconds off for 25 minutes at 90% intensity with tip with 1 cm diameter in the Digital Sonifier (*Branson*) The sample was then centrifuged for 1 hour at 100 000g. RR from the supernatant was then purified using a previously equilibrated nickel affinity column (HisTrap HP 1mL (*GE Health*). The whole protein sample was loaded on the column at 1 ml/min. The column was then washed with 25 ml of buffer containing 20 mM Tris pH8, 300 mM NaCl and 50 mM imidazole. The elution was done equally with 25 ml of buffer containing 300 mM imidazole.

Following the nickel elution, the eluate of 4 ml containing the protein was directly injected into a 125 ml SuperDex 75 size exclusion chromatography column in of a total volume of 125 ml. The nickel affinity column and the gel filtration were performed at the Membrane Protein Purification Platform (MP3) at the IBS operated by Michel Thepaut. The fractions containing the RR were concentrated using a 10 kDa MW CO PES tube (*Milipore*). Glycerol was added to the solution to a final concentration 10% (v/v) and the protein was then aliquoted, flash frozen in liquid N_2 and stored at -80 °C.

2.3.2. Membrane Extraction

Cell cultures were resuspended in 20 ml per liter of buffer containing 100 mMTris pH8, 300 mM NaCl, 20 mM $MgCl_2$ a Complete-EDTA free tablet of protease inhibitors (*Roche*) and DNase (*Qiagen*) (0.1 mg/ml). Cells were lysed using a CellDestructor (*Microfluidics*) at 18kpsi. The samples were centrifuged at 25 000 g for 30 minutes and the supernatant was then ultracentrifuged for an additional two hours at 130 000 g, then the membranes in the pellet were resuspended in a buffer containing 50 mM Tris pH8, 300 mM

NaCl, and 250 mM sucrose (2 ml buffer per 1l of culture). The membranes were then aliquoted, flash frozen in liquid N_2 and stored at -80 °C. Total membrane protein concentration was determined using the Bicinchoninic Acid Kit for Protein Determination (*Sigma*) following the manufacturer's instructions. The assay relies on the Cu^{2+} protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+} proportionally to the protein present and may be measured at OD_{562}. On average, the membrane preparation contained between 4 to 6 mg/ml of total protein.

2.3.3. ABC transporter Sample Preparation

For the ABC transporter, the screening of detergents was performed by the Robiomol Platform at the IBS. For purification, Lauryl-Maltose-Neopentyl-Glycol (MNG3) was chosen as the most suitable. Then 10 ml of total membranes extracts with ABC (40 - 60 mg) was solubilized overnight at 4 °C in presence of 1.5% (w/v) MNG3 and spun down at 110 000 g for 1 hour. The supernatant was then diluted to 0.25-0.5% MNG3 in order to avoid interference of the histidine tag binding to 1 ml Ni-NTA resin (*Qiagen*). The protein solution and nickel were gently agitated at 4 °C for 3 to 4 hours. The resin was afterwards washed with 250 ml 100mM Tris pH8, 150 mM NaCl and 50 mM Imidazole and a range of detergent to test. The proteins were eluted using 100 mM Tris pH8, 150 mM NaCl and 300 mM Imidazole and identical concentration of detergent as in the washing buffer. The best results were observed with 0.005% to 0.01% of MNG3. 300 µl of the eluate with the highest protein concentration was injected into the pre-equilibrated size exclusion chromatography SuperDex S200 10/300 GL column of a total volume of 25ml with buffer containing 100 mM Tris pH8, 150 NaCl and the same of detergent as in the injected sample. Further experiments were conducted within 72 hours after purification as the protein in detergent precipitated after storage at - 80 °C.

2.3.4. HK Sample Preparation

The detergent screen for HK was performed manually. The 4 mg of total membrane preparation were solubilized overnight at 4 °C with various detergents at 1% (w/v). The samples were then ultracentrifuged at 110 000 g for 45 minutes and the supernatant and the pellet were analyzed using a Western blot. For purification, MNG3 was selected as the most suitable detergent. The purification was performed using the Strep-Tactine® Purification protocol as directed by the manufacturer (*IBA*).

2.3.5. SDS-PAGE

For analysis, 20 µl of protein samples were supplemented with 20 µL of 2 x Laemmli blue (final concentration: 62.5 mM Tris pH 6.8, 0.4% SDS, 650 mM β-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). They were then routinely loaded on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Of note in that the samples were not boiled before electrophoresis. The electrophoresis buffer used contained 25 mM Tris, 0.1% SDS and 200 mM glycine). The proteins were revealed routinely by Coomassie blue staining. After the run, the gels were either incubated for 120 minutes in a solution of 50% ethanol, 8% acetic acid and 0.25% Coomassie R250 and destained in a solution containing 5% ethanol and 7.5% of acetic acid for 60 to 90 minutes or the gels were stained for 60 in InstantBlue^{TM} (*Expedeon*). If required, the density of the bands revealed by Coomassie blue staining was analyzed with the ImageJ freeware.

The proteins were also revealed using silver staining when indicated. After the run, the gels were activated in 50 mL acetic acid, 200 ml EtOH, 250 ml H_2O twice 20 minutes, washed in pure water twice for 10 minutes, incubated in revelatory containing sodium thiosulfate 0.2 g/ml for a minute. The gels were washed again for two minutes in pure water. Staining was done in a solution containing $AgNO_3$ 2mg/ml, 100µl 37% formaldehyde for thirty minutes. The gels were rinsed by pure water and the bands developed by 100ml solution containing 30 mg/ml K_2CO_3 , 25 µl formaldehyde, 12.5µl 10% Sodium thiophosphate until the bands were seen. The revelation reaction was stopped by placing the gels in a solution containing 25 mg/ml of glycine.

2.3.6. Western Blot

To confirm the identity of a given protein, one way is to use antibodies that specifically bind epitopes presented by the protein. The western blot is a method that allows the identification of SDS-PAGE separated proteins based on this principle. In this work, the western blots were performed using the following protocol. After SDS-PAGE, the proteins were electrotransfered (100 V, 30 min) to a nitrocellulose membrane (BioRad) in 25 mM Tris, 200 mM glycine. The membrane was saturated for one hour in phosphate buffer saline (PBS), 0.3% Tween-20, 5% milk.

For histidine tagged proteins, anti-His antibodies coupled with horse-radish peroxidase (HRP) were added at an appropriate dilution and incubated for 3 hours, prior to washing 3 times for 10 min in PBS, 0.3% Tween-20. After 3 washing steps performed as above, the detected proteins were revealed by chemo luminescence West Pico ECL kit (*Pierce*) and

exposition to a photographic film (Kodak) or in ChemiDoc (*BioRad*). All steps were performed at room temperature.

For streptavidine tagged HK, anti-streptavidine mouse primary antibodies were added at an appropriate dilution and incubated over night at 4 °C, prior to washing as above. The membrane was then incubated for 1 to 2 hours with secondary anti-mouse antibodies coupled to HRP diluted 5 000 times in PBS, 0.3% Tween-20, 5% milk. After 3 additional washing steps performed as above, the detected proteins were revealed using SuperSignal^{TM} West Femto ECL kit, (*Pierce*) and exposition to a photographic film (Kodak) or in ChemiDoc (*BioRad*).

2.4. AUC experiment

Sedimentation velocity experiments were done on an analytical ultracentrifuge XLI (Beckman Coulter, Palo Alto, USA) with a rotor speed of 42 000 rpm (Anti-60 rotor), at 4 °C, and double-sector cells of optical path length 12 and 3 mm with Sapphire windows. Acquisitions were made using absorbance at 280 and 230 nm and interference optics. The reference is the buffer used to the sample, without detergent [138]. The analysis was done with: the SEDFIT software, version 14.7g, Gussi 1.0.9g and REDATE 0.2.1. The experiment was conducted by Aline Le Roy of the IRPAS team, IBS.

2.5. ATPase activity tests

ATPase activities were measured using an enzymatic assay that allows ATP regeneration coupled to NADH oxidation, and which is followed in real-time at 37 °C at 340 nm with a UVmc2 Safas spectrophotometer or ClarioStar (BMG Labtech) [112] (Figure 3.2.). ATPase activities were measured at 37 °C in 1 ml of the activity buffer with final concentrations of 50 mM Tris-HCl pH 8, 100 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, 1.5 nM β-mercaptoethanol supplemented with 32 µg/ml lactate dehydrogenase (LDH) (*Roche*), 60 µg/ml pyruvate kinase (PK) (*Roche*), 4 mM phosphoenolpyruvate (PEP), and 0.4 mM NADH and, unless stated otherwise, 4 mM ATP. Ortho-vanadate (*Sigma*) was prepared at 200 mM as suggested by the manufacturer (*Sigma*), and heated at 95°C for 5 min before use.

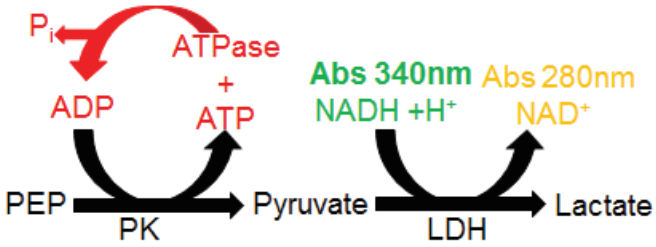


Figure 3.2. ATPase assay reaction. After the protein uses ATP, the pyruvate kinase (PK) recharges ADP to ATP using a phosphate from phosphoenolpyruvate (PEP). The resulting pyruvate is further hydrogenated from NADH by lactate dehydrogenase (LDH) to lactate. NADH absorbs at 340nm and NAD^+ at 280nm, therefore we may follow the disappearance of signal. For 1 mol of NADH oxidization, 1 mol of ATP has been used.

To calculate the specific activity the following equation (Lambert's law) is followed:

$$\Delta OD = \varepsilon . l . \Delta c$$

Where $\varepsilon NADH_{340nm} = 6220$ (l/mol/cm), l = volume of the assay (1 ml in our case) and Δc = rate of NADH disappearance (mol/min). Therefore the rate may be calculated:

$$\text{Rate (nmol/min/mg)} = \frac{\Delta DO\ (min^{-1}) \times 1000\ \mu l\ .\ 10^3}{6220 \times \text{Protein (in } \mu g)}$$

2.7. Insertion of BceAB type ABC transporter into nanodisc

The following protocol for ABC transporter reconstitution in nanodisc was adapted from [202]. Briefly, *E. coli* polar lipid extract (*Avanti Polar Lipids*) were well dried overnight in vacuum to remove excess chloroform in which they were dissolved. Then we reconstituted them in a buffer containing 20 mM Tris pH 7.4, 100 mM NaCl and 0.086% DDM. Then we mixed together to a final volume of 5 ml the samples in the following ratio 1.5 mg protein, 10 mg lipids and 10 mg MSP (MSPE3D1, courtesy of Yann Huon de Kermadec). The mixture was agitaded for one hour at room temperature. Then, to remove the detergent, 650 mg/ml biobeads (*BioRad*) were added and the mixture was shaken for an additional two hours and the biobeads were afterwards removed by filtering the sample. The samples were then placed at 4°C and we for purification, performed size exclusion chromatography in the S200 10/300 column of total volume of 25 ml (*GE HealthCare*). The filtration fractions were then analyzed by SDS-PAGE gels.

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Abstract

Streptococcus pneumoniae, the pneumococcus, is a major human pathogen causing over a million deaths each year. Many pneumococcal strains display resistance towards antibiotics causing world-wide health concern. Some of these antibiotics are antimicrobial peptides (AMP), which are produced as a primary defense by hosts as well as pathogens. The pneumococcus harbors a system comprised of an ATP-binding cassette (ABC) transporter and a two-component system (TCS) composed of a histidine kinase (HK) and a response regulator (RR), which targets these molecules. It has been shown recently that the removal of this ABC transporter increases the sensitivity of the bacteria towards bacitracin. In this project, we tried to understand the functioning mechanism of the ABC transporter and the co-operation with the TCS using both *in vivo* and *in vitro* techniques.

Résumé

Streptococcus pneumoniae, le pneumocoque, est un pathogène majeur causant plus d'un million de morts par an dans le monde. De plus en plus de souches de pneumocoques sont résistants aux antibiotiques, en faisant un problème majeur de santé publique dans le monde. Une partie des ces antibiotiques sont les peptides anti-microbiens (AMP), qui sont produit aussi bien par l'hôte que des bactéries pathogènes en tant que premier système de défense. On trouve dans le pneumocoque un transporteur ABC (ATP-Binding Cassette) lié à un système de deux composants (TCS) – la kinase d'histidine (HK) et le régulateur de réponse (RR), qui cible les AMP. Récemment, il a été démontré, que l'absence du transporteur ABC augmente la sensibilité à la bacitracine. Dans ce projet, nous avons essayé à comprendre le mécanisme fonctionnel entre le transporteur ABC et TCS en utilisant des outils *in vivo* et in *vitro*.