Cells employ membrane proteins to transport molecules in and out of cells. A class of these proteins, known as ATP-binding cassette (ABC) transporters, are powered by ATP hydrolysis to export or import molecules against their concentration gradient. Prokaryotic ABC transporters involved in solute uptake use substrate-binding proteins (SBPs) to recognize, capture and eventually deliver the solutes for translocation by the ABC transporter. Different types of SBP-dependent ABC importers are shown in Figure 1. Members within the ABC superfamily, most notably the OTCN (osmoprotectants, taurine, cyanate and nitrate) and the PAO family (polar amino acids and amines), can have SBPs fused to the C- or N-terminus of the transmembrane domain (TMD) [Figure 1; panel C and D, respectively]; SBPs fused to the TMD are referred to as substrate-binding domains [SBDs]. From one to six SBDs have been found per functional ABC transporter complex. ABC transporters with the SBDs fused to TMD are present mostly in Gram-positive bacteria, including pathogens such as *Listeria monocytogenes, Staphylococcus aureus, Streptococcus pneumoniae, Enterococcus faecalis, Streptococcus pyogenes* and others.

![Figure 1. Cartoon representation of SBP-dependent ABC importer. (A) In Gram-negative bacteria (e.g. *E. coli*) SBP is mostly present as soluble periplasmic protein. In Gram-positive bacteria, SBP is anchored to the lipid membrane (B) or fused to the translocator domain (C) and (D). (D) GlnPQ consists of two times two subunits. Subunit P corresponds to two SBDs fused to the N-terminus of the TMD. Subunit Q corresponds to the nucleotide-binding domain.](image)
Previously, we have shown that the fusion of (multiple) SBDs near the transmembrane domain (TMD) enhances the activity of substrate transport \(^3\). In this study, we show that multiple SBDs also broaden the substrate specificity of the transporter. This thesis focuses on GlnPQ, which has two different SBDs arranged in tandem, fused to the N-terminus of the translocator domain (Figure 1; panel D). GlnPQ is an amino acid importer that is specific for glutamine, glutamic acid and asparagine (Figure 2).

![Glutamic acid, Glutamine, Asparagine](image)

*Figure 2. Different substrates of GlnPQ.*

**Structural and functional characterization of the substrate-binding domains of GlnPQ**

![X-ray crystallography](image)

*Figure 3. Structures of substrate-binding domains of GlnPQ were solved by X-ray crystallography. All structures were solved by the molecular replacement method, using high-quality X-ray diffraction data. For example, to solve the structure of the unliganded-SBD2 from *L. lactis*, the structure of the glutamine-binding protein GlnH from *E. coli* was used as a search model (PDB code: 1GGG). The obtained SBD2 structure was then used as the search model to solve the other SBD structures reported in this thesis.*

In chapter 2 and chapter 3, we present the crystal structures of the SBDs of GlnPQ from non-pathogenic and pathogenic Gram-positive bacteria; structures were solved in open and closed-liganded conformations. Even though the SBDs
sequences diverge by 50%, the overall fold is preserved. The SBDs consist of two globular subdomains connected by a hinge that is formed by two anti-parallel β-strands. Upon ligand binding the two sub-domains rotate by \( \sim 20° \) around the hinge and engulf the ligand in the crevice between the two subdomains. The binding sites of the SBDs of GlnPQ from *Lactococcus lactis*, *Enterococcus faecalis*, and *Streptococcus pneumoniae* have a similar architecture, but the specificity and affinity for substrates is different. Close inspection of the binding sites suggested a small number of residues that might be responsible for the difference in specificity and affinity. By using a mutagenesis approach and structure modelling, we succeeded in converting a low-affinity SBD into a high-affinity receptor and *vice versa*. Moreover, we show that the SBDs in the tandem have a different specificity, which allows a cell to accumulate different amino acids via a single ABC transporter.

**Substrate competition in GlnPQ and its implication for growth of *L. lactis***

GlnPQ from *L. lactis* has relatively high affinity and low rate of transport for asparagine via SBD1, and the opposite is true for glutamine and glutamic acid. On the other hand, SBD2 has high affinity for glutamine and fast transport of this amino acid. While asparagine binding to SBD2 could not be detected, transport activity was observed when asparagine was supplied at submillimolar concentrations. In Chapter 4 we address the competition between these amino acids for import via GlnPQ and its consequences for growth of *L. lactis*. We characterize the growth of cells in terms of lag phase (\( \lambda \)), growth rate (\( \mu \)) and biomass yield (here indicated as \( \text{OD}_{\text{max}} \)) (Figure 4; panel A). Figure 4; panel B shows an example of competition between glutamine and asparagine uptake via SBD1, which is reflected in the growth of *L. lactis*. When the growth medium is supplied with equimolar concentrations of asparagine and glutamine, uptake of glutamine via SBD1 is strongly inhibited by asparagine and the growth rate is slow (red line). By increasing the glutamine concentration, uptake of glutamine via SBD1 and thus the growth could be restored (blue and magenta).

Finally, we have shown that GlnPQ has a higher transport capacity for glutamine than for asparagine. This is in line with the higher requirement for glutamine (and glutamate) than asparagine, and the role of glutamate in cell volume regulation \(^4\). We note that inside the cell most of the glutamine is readily converted into glutamate and the pools of these amino acids do not differ much when either glutamine or glutamic acid is transported.
Summary

Figure 4. (A) Bacterial growth is characterized by a lag phase ($\lambda$), growth rate ($\mu$) and biomass yield ($OD_{max}$). (B) Growth of *L. lactis* carrying GlnPQ-SBD1 in CDM* medium supplied with 3 mM of asparagine plus different concentration of glutamine (black= 0, red = 3 mM, blue = 10 mM, or magenta = 30 mM).

**SBDs of GlnPQ as potential target for antibacterial drug-development**

GlnPQ is required for nitrogen source intake and the system has been implicated in virulence in pathogenic bacteria. Importantly, GlnPQ lacks homologs in humans and mammals, and is therefore a potential target for drug development. In **Chapter 5**, a strategy to develop drugs against pathogenic Gram-positive bacteria is described, using the structural knowledge of SBDs obtained in chapter 2 and 3 and growth assay presented in chapter 4. We combined virtual screening methods of ligand docking with a high throughput bioactivity assay to identify first lead compounds that affect growth when the target organism is made dependent of transport via GlnPQ. Our strategy is simple and readily discriminates GlnPQ effectors from non-specific inhibitors by comparing growth on glutamine (or glutamate) *versus* alanyl-glutamine (Figure 5). The results of this study offer an alternative strategy to fight pathogenic bacteria through the inhibition of an essential ABC transporter.
Figure 5. The requirement of glutamine in *L. lactis* can be fulfilled by glutamine (grey dots) uptake via GlnPQ (cartoon in orange) [A] or glutamine-containing peptide import (blue dots) via DtpT (cartoon in green) [B]. When an inhibitor (red) of GlnPQ is present in the medium, uptake of glutamine is blocked, resulting growth inhibition (C). However, the same inhibitor does not inhibit growth when the medium is supplemented with a glutamine-containing peptide, which is transported via DtpT (D). If the inhibitor affects the cell non-specifically (e.g. by affecting the membrane permeability or metabolic energy conservation) then growth is reduced both in the presence of glutamine and glutamine-containing dipeptide (E). (F) Schematic overview of structure-based virtual screening. Structures of SBDs of GlnPQ were used as template to virtually dock hundreds of thousands of small molecules. The hit compounds derived from this virtual screening were tested in high throughput growth assay. Potential lead compounds, i.e. those inhibiting growth in the presence for glutamine but not in the present of glutamine-containing peptides, were tested further in transport assays with GlnPQ-expressing cells.
Summary

The work presented in this study shows how a combination of structural and functional studies can be used to better our understanding of how a biological transport system works. Considering the advantage of having SBDs fused near to the translocator domain (more efficient transport), it is surprising that this feature is not more common within the ABC importers. Yet, this type of transporter is undoubtedly important for bacterial survival.

References


