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Structure–function analysis of the ATP-driven glycolipid efflux pump DevBCA reveals complex organization with TolC/HgdD

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ABSTRACT

In Gram-negative bacteria, trans-envelope efflux pumps have periplasmic membrane fusion proteins (MFPs) as essential components. MFPs act as mediators between outer membrane factors (OMFs) and inner membrane factors (IMFs). In this study, structure–function relations of the ATP-driven glycolipid efflux pump DevBCA-TolC/HgdD from the cyanobacterium *Anabaena* sp. PCC 7120 were analyzed. The binding of the MFP DevB to the OMF TolC absolutely required the respective tip-regions. The interaction of DevB with the IMF DevAC mainly involved the β-barrel and the lipoyl domain. Efficient binding to DevAC and TolC, substrate recognition and export activity by DevAC were dependent on stable DevB hexamers.

Structured summary of protein interactions:
DevB binds to **DevB** by surface plasmon resonance (View interaction) **DevC** and **DevA** physically interact with **DevB** by surface plasmon resonance (View interaction) **TolC** binds to **DevB** by surface plasmon resonance (1, 2) **DevB** and **DevB** bind by molecular sieving (View interaction)

1. Introduction

Gram-negative bacteria use tripartite trans-envelope efflux pumps/type 1-secretion systems to export a wide variety of molecules [1–5]. These exporters span the cytoplasmic membrane, the periplasm and the outer membrane. They are composed of inner membrane factors (IMFs) and of outer membrane factors (OMFs), and both are connected by central periplasmic membrane fusion proteins (MFPs, Fig. 1A) [2,3].

IMFs can either be ATP-driven (ATP-binding cassette (ABC) superfamily, also known as type I secretion systems) or gradient-driven (resistance-nodulation-division (RND) or major facilitator superfamily), while TolC-like OMFs can promote efflux beyond the outer membrane by the different IMFs [1–7] (Fig. 1B). The periplasmic MFPs differ from each other in sequence, molecular mass and biochemical properties, but are structurally similar [8]. A typical ABC-type MFP consists of the following structural elements: an N-terminal cytoplasmic tail, a cytoplasmic membrane spanning anchor helix, a β-barrel domain, a lipoyl domain and an α-helical domain protruding toward the OMF (Fig. 1A and D) [2,9,10].

Most of our knowledge on tripartite efflux systems is based on studies on the RND-type multidrug efflux pump AcrAB-TolC [2,11,12], and on studies on the ABC-type efflux pump MacAB-TolC [1,2,13]. Evaluation of data derived from cross-linking experiments indicated a trimer of the MFP AcrA connecting the IMF AcrB and the OMF TolC [14–17]. The AcrA trimer binds with its α-helical domains to the α-helical barrel of TolC in a coiled-coil like manner. The MFP MacA is assumed to connect the IMF MacB and TolC by forming a hexamer [18–20]. MacA and TolC interact in a cog-wheel-like assembly between both tip-regions of the respective α-helical domain.

Our previous work on DevBCA-TolC supported the proposed model of the MacAB-TolC efflux pump [21]. The IMF DevAC, the MFP DevB and the OMF TolC, also known as HgdD, from the filamentous cyanobacterium *Anabaena* sp. PCC 7120 were shown to form an ATP-driven efflux pump to export glycolipids in the course of heterocyst maturation [21]. Heterocysts are formed during depletion of combined nitrogen in a semi regular pattern along the filaments of this photoautotrophic bacterium. Whilst the vegetative cells perform oxygenic photosynthesis, the heterocysts are specialized for fixation of dinitrogen by nitrogenase. These specialized cells provide a micro-oxic environment suitable for nitrogenase and they exchange metabolites with the vegetative

Abbreviations: ABC, ATP-binding cassette; IMF, inner membrane factor; MFP, membrane fusion protein; OMF, outer membrane factor; RND, resistance-nodulation-division; SEC, size exclusion chromatography; SPR, surface plasmon resonance

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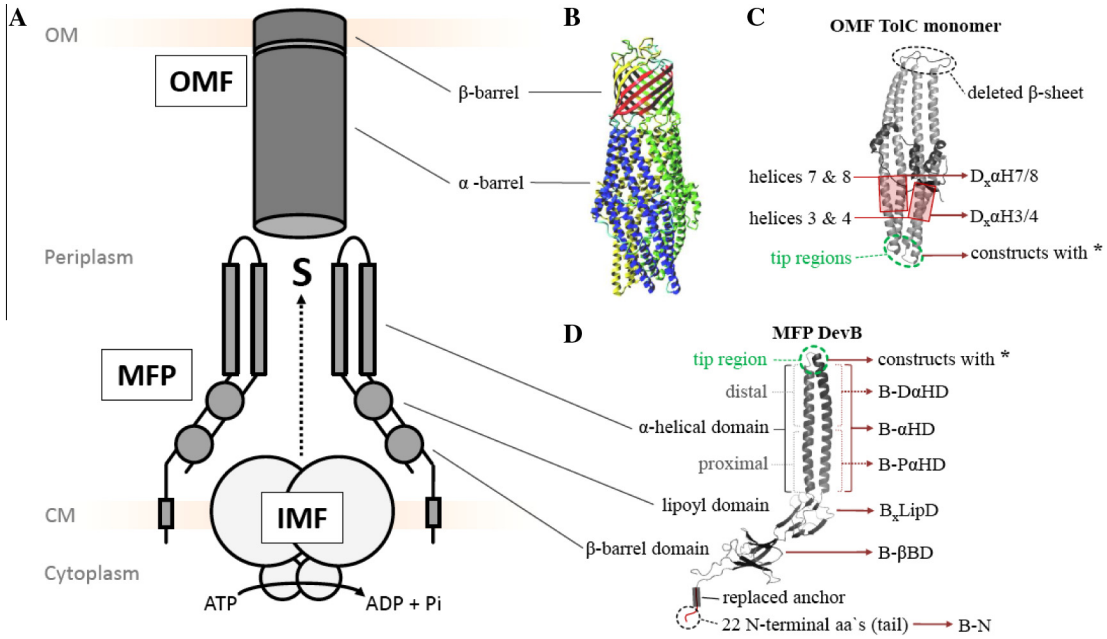

Fig. 1. ATP-driven efflux pumps and variants of DevB and TolC used in this study. (A) Common model of an ATP-driven efflux pump/type 1 secretion system. IMF = inner membrane factor, MFP = membrane fusion protein, OMF = outer membrane factor, S = substrate, CM = cytoplasmic membrane, OM = outer membrane. (B) Theoretical quaternary structure of TolC from Anabaena [28]. (C) Illustrating model of one monomer of *Anabaena's* TolC based on resolved crystals of TolC from *E. coli* (DOI: 10.2210/pdb1ek9/pdb). Red arrows indicate the construct name and modifications (listed in Table 1). (D) Illustrating model of *Anabaena's* DevB based on resolved crystals of MacA (DOI: 10.2210/pdb3fpp/pdb) from *E. coli.* The much longer α-helical domain was not considered in the model (compare sequences in Fig. S7). Red arrows indicate the construct name and modifications (listed in Table 1 and Fig. S2).

cells [22–24]. Among other adaptations, heterocysts deposit two additional layers on the top of the cell wall to restrict the entrance of oxygen from the aerobic environment. The innermost layer is made up of heterocyst-specific glycolipids stabilized by the outermost polysaccharide layer [25]. In our previous study, we showed that the DevBCA-TolC system exports the heterocyst glycolipids [21,26–28]. The crucial stoichiometric relations of DevBCA-TolC for export were found to be in line with the ones derived for MacAB-TolC: the IMF-to-MFP-to-OMF ratio was calculated to be 2:6:3 [21]. In this work, we addressed the key role of the MFP DevB. By using size exclusion chromatography (SEC), surface plasmon resonance (SPR), and ATP hydrolysis assays, we investigated the influence of modified variants of DevB on both the intra-pump interactions and the activity of DevBCA-TolC. This structure–function analysis contributes to a better understanding of the assembly and the function of an ATP-driven efflux pump.

2. Materials and methods

2.1. Construction, overexpression, and purification of recombinant proteins

Proteins were overexpressed and purified as described previously [21]. In brief, all protein variants were overexpressed as glutathione-S-transferase (GST) tag fusions in *Escherichia coli* strain Rosetta-Gami™ DE3 using cloning vector pET42a (Merck, Darmstadt, Germany). The plasmid constructs are listed in Table S1; the oligonucleotides used for amplification are listed in Table S2. Recombinant proteins were purified using GST SpinTrap or GSTrap FF columns (GE Healthcare). The N-terminal GST tag was cleaved off by using Factor Xa and removed. The protease was removed by using Xa Removal Resin (Qiagen). SDS–PAGE slices of the purified proteins are shown in Fig. S1. A register of the protein variants is presented in Table 1, a graphical representation in Fig. 1C and D and important aa sequence variations are shown in Fig. S2.

2.2. Gel filtration chromatography

Recombinant proteins were analyzed via size-exclusion chromatography (SEC) as described previously [21]. In brief, 0.7 and 1 mg/ml of TolC and DevB proteins, respectively, were separated on a Superdex 200 HR 10/30 gel-filtration column (GE Healthcare) in running buffer (25 mM MES-NaOH, pH 6.2; 150 mM NaCl and 0.05% Triton X-100) at a flow rate of 0.5 ml/min.

2.3. Surface plasmon resonance spectroscopy

Surface plasmon resonance (SPR) experiments were performed by using a Biacore X biosensor system (Biacore AB, Uppsala, Sweden) as described previously [21]. His-tagged baits were immobilized in flow cell 2 (FC2) of a Ni^{2+}-loaded NTA sensor chip, and His tag-free preys in reaction buffer (25 mM MES-NaOH at pH 6.2; 150 mM NaCl; and 0.05% Triton X-100) were injected into FC1 and FC2 at a flow rate of 20 µl/min. Specific interactions were captured as response difference between FC2 and FC1. To avoid preformation of higher oligomers of reference DevB on the chip surface, lower concentrations of immobilized reference DevB have been used (~80 and 65 RU instead of ~910 RU of immobilized DevB in assays with TolC; compare Figs. S4 and S5B).

2.4. Cell fractioning and glycolipid purification

Cell fractions [28] and pure heterocyst glycolipids [29] were prepared as described. In brief, enriched heterocysts [28] were broken by at least five passes through a French Pressure cell (24000 psi) and separated into a soluble cytoplasmic and an insoluble membrane fraction by centrifugation (45000*g*, 30 min, 4 °C). The pellet was separated by the respective gradients to purify cell fractions or heterocyst glycolipids [29].