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Full Length Article

Expression and Analysis of Two-partner Secretion System of *Neisseria meningitidis* using Heterologous Host *Escherichia coli*

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Abstract

In Two-Partner Secretion (TPS) systems, the outer membrane-embedded TpsB transporter is dedicated for the transport of a large exoprotein, generically called TpsA. However, its mechanism of transport across the outer membrane is not fully understood. Expression and production of neisserial TPS systems in ordinary laboratory host strains such as *Escherichia coli* MC1061 is highly desirable for convenient handling and reduced hazards. Therefore, in the current study, we reconstituted TPS-2of *N. meningitidis* by cloning TpsB2 transporter protein downstream to its substrate TpsA protein in pET expression systems to constitute an articulated TPS system for expression in heterologous host *E. coli* MC106. Our results indicate that TPS system 2 secretes its TpsA substrate as efficient as like wild type host *N. meningitidis*. The secretion was isopropyl-β-D-thiogalactopyranoside (IPTG) dose dependent up to some level and TpsB2 was properly integrated, folded and stably operated in the outer membrane as like in the wild type host *N. meningitidis*. The tool will be useful to further investigate and unravel the molecular determinants involved during initial interaction and the most importantly for large scale production of the recombinant TPS components for downstream procedures such as crystallography and protein –protein interaction studies.

Keywords: Heterologous protein expression; *Neisseria meningitidis*; Two-partner secretion systems; TpsA; TpsB

Introduction

In Gram-negative bacteria, the Two-Partner Secretion (TPS) system is a distinct subclass of the Type V secretion pathway, which also includes the classical and trimeric autotransporters systems consist of minimally a large TpsA protein that is secreted by a dedicated TpsB outer membrane (OM) transporter (Schmitt *et al.*, 2007; Neil and Apicella, 2009; Grijpstra *et al.*, 2013; van Ulsen *et al.*, 2014). TpsA proteins are typically over 100 kDa and have a predominantly β-helical structure (Kajava *et al.*, 2001; Kajava and Steven, 2006). TPS proteins have been reported to act as adhesins, toxins for eukaryotic or bacterial targets and receptors/scavengers of nutrients from the environment (van Ulsen *et al.*, 2014; Piet *et al.*, 2016). The TpsB transporter secretes the TpsA protein across the outer membrane in a process that is not fully understood. The main components, TpsA and TpsB, of TPS system are synthesized in the cytoplasm carrying an N-terminal signal peptide by which are transported over the inner membrane into the periplasmic space via the Sec translocon. TpsB subsequently integrates in the OM and interacts with the N-terminal domain of TpsA protein (~250-300 residues and also called the TPS domain) that initiates its secretion (Jacob-Dubuisson *et al.*, 2001).

The OM TpsB proteins is a member of Omp-85 super family, which contains the BamA protein implicated in the biogenesis of OM-based β-barrel proteins . This family member proteins are characterized a by a 16-stranded β-barrel at the C-terminus preceded by a periplasmic soluble module that includes one to five POTRA (polypeptide transport-associated) domains as indicated by crystal structure of TpsB, FhaC (Clantin *et al.*, 2007; Gatzeva-Topalova *et al.*, 2008; Arnold *et al.*, 2010). POTRA domains interact with the target proteins of their transporter (Knowles *et al.*, 2008) and it has been shown that the POTRA domains interact with the TPS domain and its deletion render TpsB inactive (the pro-protein form of secreted FHA) at least *in vitro* (Hodak *et al.*, 2006; Delattre *et al.*, 2011).

N. meningitidis, the Gram-negative diplococcous, is a major cause of meningitis and sepsis world-wide (Stephens *et al.*, 2007). *N. meningitidis* genomes encode up to three TPS systems, systems1-3 (Fig. 1), and the system has been implicated in pathogenesis (van Ulsen and Tommassen, 2006; van Ulsen *et al.*, 2008). We recently showed that TpsB1 of system-1showed substrate specificity, whereas TpsB2 was shown to secrete TpsAs of other TPS systems as well (Sadeeq-ur-Rahman and van Ulsen, 2013; Sadeeq-ur-Rahman *et al.*, 2014). It has been shown previously that system specificity is conferred by the periplasmic POTRA domains (Sadeeq-ur-Rahman *et al.*, 2014). However, the molecular determinants that play a role in initiation of interaction and specificity between the POTRA domain and TPS domain is not known yet. To get further insight into this, one of the approaches would be to synthesize purified high amount of both POTRA and TPS domain that can further be crystallized. This is highly desirable in a common laboratory nonpathogenic host such as *Escherichia coli*. Here, we report on such a heterologous system based on nonpathogenic *E. coli* strain that can be conveniently used for analysis of TPS system of *N. meningitidis*.

Materials and Methods

Growth and Bacterial Strains

The *N. meningitidis* and *E. coli* strains utilized for this study are given in Table 1. *N. meningitidis* strains were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C, 5% CO_2. Tryptic soy broth (Gibco-BRL) was used for liquid cultures of *N. meningitidis* that was grown at 37°C and was supplemented chloramphenicol to a final concentration of 8 µg/mL for selection of plasmid. *E. coli* strains were grown on lysogeny broth (LB) or grown on LB agar plates supplemented with 100 µg/mL ampicillin for plasmid maintenance and with 0.5% glucose for full repression of the *lac* operator, when appropriate.

Cloning and Construction

TpsB2 gene was PCR amplified from the genomic DNA of *N. meningitidis* using a set of primers (pr_tpsB2_F:CACATATGGATCCGTGTATTGAATGCC CATTGATGA, pr_tpsB2_End: GAGATCTGAATTCCGTTGATTTGACTATGCCGTTT TA) (Under line sequences represent restriction enzyme sites used for cloning) and high fidelity DNA polyermerease (Roche) from chromosomal DNA of HB-1 *N. meningitidis* and the resulted amplicon was cloned into pGEMT (Promega) (Table 1), that was then confirmed by sequencing (Macrogen) (Sadeeq-ur-Rahman and van Ulsen, 2013). The ORF *tpsB2* was then clones in front of the TPS domains, a truncated *tpsA2* (TPS2) (Sadeeq-ur-Rahman and van Ulsen, 2013) in pET11a using BamHI/EcoRI restriction sites (Fig. 1).

Western Blotting and SDS-PAGE

All procedures were carried out as described earlier (Sadeeq-ur-Rahman *et al.*, 2014). Briefly, cultures of *N. meningitidis* HB-1 were grown for 4-5 h to an OD_{600} of ~2.5.0-3.5 with 1- to 0.1 m*M* of isopropyl-β-D-thiogalactopyranoside (IPTG) when indicated. Centrifugation (4,500×*g*, 5 min) was used to pellet down bacterial cells for harvesting. Bacteria pellet was resuspended in phosphate-buffered saline pH 7.4 (PBS) maintaining concentration to a final OD_{600} of 10. Residual cells were removed by centrifugation (16,000×g, 10 min) of supernatant. Secreted proteins were precipitated from the supernatant samples using 5% trichloroacetic acid (TCA) and dissolved in a volume of PBS corresponding to a cell density of OD_{600} 100 (10× concentrated compared to cells). Samples were then mixed 1:1 with. 2× sample buffer (mix). *E. coli* culture expressing TPS system 2 or 3 through pET plasmids were grown in LB broth to exponential phase (OD_{600} of ~0.4-0.6) at which point induction was carried out by adding IPTG to a final concentration of 0.1- to 0.5- m*M* and incubation was resumed for another 1-2 h. Samples were collected as described above. Protein samples were separated on 7.5-10- or 12-% SDS-PAGE gels (BioRad) and proteins were blotted onto nitrocellulose for western-blot analyses. Blots were treated as described earlier and incubated anti-TPS1 and anti-TPS2a or anti-TpsB1 and anti-TpsB2 (Sadeeq-ur-Rahman and van Ulsen, 2013). The secondary antibody used was goat anti-rabbit immunoglobulin G serum that was conjugated to horseradish peroxidase (Biosource International). The binding of TPS protein with conjugated antibodies on the blots was visualized applying Lumilight- normal or Plus (Roche). The relative molecular weight of proteins indicated on each blot or SDS-PAGE was deduced from the Precision Plus Protein Standard (BioRad) run in each SDS-PAGE gel.

Outer Membrane Isolation and Heat Modifiability

Outer membrane fractions were isolated according to (Sadeeq-ur-Rahman *et al.*, 2014). Cells were harvested by centrifugation (4,500×*g*, 5 min). The pellet was resuspended in a 50 m*M* Tris-HCl pH 8.0 and 2 m*M* EDTA buffer and was passed 2 times through a One Shot Cell disrupter (Constant Systems Ltd) at 30,000 psi or sonicated. Unbroken cells were pelleted by centrifugating the lysate (4,500×*g*, 5 min). Crude membranes were obtained by ultracentrifugation of the contents (200,000×g, 30 min) and were resuspended in PBS. Denaturation of crude membranes was achieved by dilution in 2×sample buffer followed by boiling for 10 min. Semi native membranes were diluted in 2× semi-native sample buffer (*i.e.* 2× sample buffer with 0.4% SDS and lacking DTT) and kept at room temperature for 10 min. All samples were then run on semi-native PAGE gels keeping temperature at lower. Western blotting for the membranes was performed as described above.

Table 1: Strains and plasmids/constructs used in this study

Strains and Constructs	Plasmid name	TPS ORFs	Reference
N. meningitidis strains			
HB-1 *tpsB1::kan tpsB2::gen*integrated with TPS-2			(Sadeeq-ur-Rahman and van Ulsen, 2013)
E. coli strains			
Top10F			Invitrogen
MC1061			(Casadaban and Cohen, 1980)
Plasmids			
TPS2a+TpsB2	pETTPS-2	*tpsA2a*-tr + *tpsB2*	this study
TPS2a	pETTpsA2	*tpsA2a*-tr	(Sadeeq-ur-Rahman and van Ulsen, 2013)
Cloning vectors			
	pGEM-T		Promega
Expression vector	pET11a		Novagen

[a]The suffix "–tr" indicates that the construct comprises a truncated *tpsA* ORF that encodes the signal peptide and the TPS domain

Results

Expression and Secretion Analysis of TPS-2 in Wild Type and Heterologous *E. coli* Hosts

To express and analyze TPS-2 of *N. meningitidis* in nonpathogenic laboratory strain such as *E. coli* MC1061, we cloned TpsB2 downstream to its truncated TpsA (TPS2a) substrate protein in pET expression system (Fig. 1A). The pET plasmid has an IPTG inducible T7 promoter downstream to which the TPS-2 system was cloned. The final construct (pETTPS2) was then transformed to electro-competent *E. coli* MC1061 cells by heat shock method. Successful transformants were selected on ampicillin containing LB agar plates. Of each independent clone, three colonies were further screened for expression and secretion analysis. To express the TPS-2 system, IPTG (100 m*M*) was added to the growing cells at log phase of the cell growth. We then collected the cells after two h of expression. Whole cell lysates and culture supernatant was then analyzed for the presence of TpsB2 and TPS2a, respectively. Western blot analysis indicates that indeed the TpsB (~62 kDa band size) was expressed in the cell pellet. Similarly, by immunoblot analysis we also showed that the TPS2a was secreted into the medium indicated by an expected ~36 kDa band size (Fig. 1B).

Effect on Growth Pattern and Cell Viability

To show that proteins of *N. meningitidis* when expressed in heterologous host, *E. coli* is not associated with toxicity and thus does not lead to cell death, we checked the growth pattern of wild type *E. coli* MC1061 expressing recombinant TPS-2. Growth curve analyses indicated that cells were growing normal as like wild type (without plasmid expressing TPS-2) and uninduced cells indicating no detectable adverse effects (Fig. 1C).

Comparison of Expression in the Wild Type Host

We then compared the expression and secretion of TPS2a in the wild type host *N. meningitidis* and heterologous expression host *E. coli* MC1061 (Fig. 2A). Our results showed that indeed the amount of TpsB2 expressed in the cell pellet and the amount of secreted TPS2a in the supernatant of heterologous *E. coli* host was comparable to the wild type host *N. meningitidis*.

Localization of TpsB Transporter in the Outer Membrane

Next we wanted to know whether TpsB transporter is properly integrated into outer membrane. For this purpose outer membranes were isolated from induced cells and analyzed for heat modifiability both in wild type as well heterologous *E. coli* host. Outer membrane proteins once integrated and properly folded show a particular heat-modifiability pattern, indicated by a folded and faster running species that can be linearized by adding reducing agent such Dithiothreitol (DTT). As expected, our results indicated that TpsB2 in heterologous host *E. coli* MC1061 was indeed properly localized and showed heat modifiability features of wild type when grown in natural host *N. meningitidis* (Fig. 2B).

Optimization of Secretion

We then determined the optimal dose and duration of induction for optimal production. For this purpose, different amounts such as 0, 50, 100, 200, 500 m*M* of IPTG was added and cells were grown for maximum of 2 h after the induction point. Our results indicated that production of recombinant protein components were IPTG dose dependent, however, keeping in mind the growth and fitness of the cells, 100 m*M* IPTG gave better outcomes (Fig. 2D). It is also known that duration of induction can also affect the amount and availability including the quality of recombinant proteins. For this purpose, we induced *E. coli* MC1061 cells at log phase for various durations such 10, 30, 60 and 120 min using 100 m*M* IPTG. Our results indicated that 2 h of total induction produced maximum proteins (Fig. 2C). Overall, our results indicate that heterologous expression of neisserial TPS-2 systems in *E. coli* MC1061 show the features of wild type and thus can be in fact used for secretion analysis and TPS characterization.

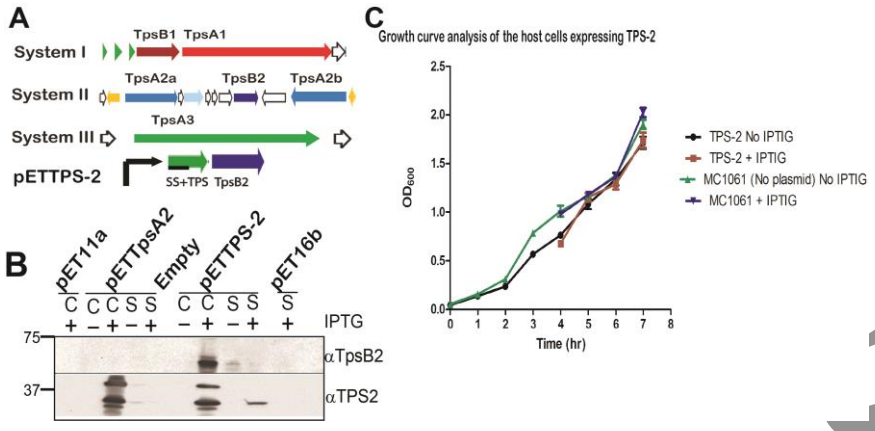

Fig. 1: Construction and expression analysis of TPS-2 system of *N. meningitidis* in heterologous host *E. coli***A.** organization of TPS system components of *N. meningitidis* into three distinct classes System 1-3. (Top) Cartoons of chromosomal localization and distribution of TPS associated genes in the chromosome of *N. meningitidis*. The size and position of the TPS associated genes are not drawn to scale and these represent approximate position and size. (bottom) Dashed line below TpsA3 indicates the approximate size of the signal sequence plus TPS domain that was cloned. (b) Expression and western blot analysis of the TPS-2 of *N. meningitidis*in *E. coli*. *E. coli* cells were transformed with pETTPS-2 and induced at log phase for a hr. cell pellet and culture supernatant was then blotted against specific sera for identification of TpsA and TpsB as shown (c) Growth curve of E. coli cells expressing TPS-2 of *N. meningitides*

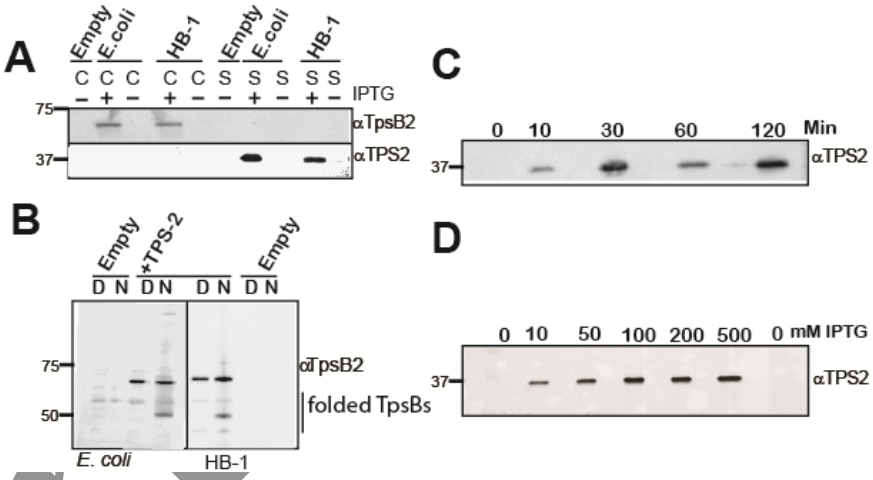

Fig. 2: Expression and optimization of TPS-2 systems of *N. meningitidis* in *E. coli* strain. **A.** Immunoblots of whole cell lysates of *N. meningitidistpsB1::kan/tpsB2::gen* expressing TPS-2 and *E. coli* cells carrying plasmids encoding TPS-2 of *N. meningitidis* as indicated above the lanes. Samples were induced (+) or not (-) with IPTG as indicated above. C is cell lysates while S is culture supernatant. **B.** Immunoblots of outer membranes isolated from induced cells of *N. meningitides* expressing TPS-2 system as wild type host or *E.coli* expressing TPS-2 system of *N. meningitidis*as in heterologous host as indicated above the lanes. Outer membranes were incubated with DTT and boiled (D= denatured) or without DTT and unboiled (N= native) (**C&D**) immunoblot of culture supernatant expressing TPS-2 systems of *N. meningitidis* in *E. coli* with different amounts of IPTG and duration of induction as indicated above the lanes. The folded proteins will run faster as compared to the denatured proteins that has been marked at expected size. The blots were incubated with the antisera indicated. Relative size of the protein as indicated on the left was obtained from running precision plus molecular weight protein (Roche) alongside samples

Discussion

TPS systems got a lot of attention in the recent years due to its conservation in pathogenic and non-pathogenic Gram negative bacteria and its diverse role in the virulence thereof. In addition to its rule in biofilm formation, intracellular survival and escape from infected cells; the killing effect of TpsA secreted proteins by contact dependent growth inhibition speeded up research to understand the mechanism of secretion of TPS systems and

its role in pathogenesis (van Ulsen *et al.*, 2014). In addition, TpsB transporter being a member of a super family protein BamA, which plays imperative role in biogenesis of outer membrane proteins has further inspired biologists to understand TPS systems (Arnold *et al.*, 2010; van Ulsen *et al.*, 2014). Furthermore, secreted proteins are generally crucial for bacteria and can be utilized for acquisition of essential elements from the environment, development of antimicrobial resistance and establishment of host-microbe interaction for initiation of infection (van Ulsen *et al*., 2014; Adnan *et al*., 2017; Ali *et al*., 2017; Ali *et al*., 2017; Sadeeq-ur-Rahman *et al*., 2018). To avoid hazards and to facilitate open bench work on neisserial TPS systems, expression and optimization of neisserial protein in heterologous common laboratory strain such *E. coli* is highly needed. In this study, we have successfully reconstituted TPS-2 systems of *N. meningitidis* in *E. coli* by achieving its optimal secretion.

The Mechanism of outer membrane integration and translocation has not been fully understood and so far BamA and TpsB FhaC proteins have been used as model proteins for these kind of studies (Noel *et al.*, 2012). Based upon the FhaC crystal structure, (Clantin *et al.*, 2007) the TpsB model suggests that POTRA domains are exposed to the periplasm (van Ulsen *et al.*, 2014). It has been postulated that initial interaction is most probably electrostatic via β augmentation, however, the precise role of the residues and interaction interface is not know yet. Since, *N. meningitidis* is a transitory colonizer of human epithelium; therefore, expression of its proteins and secretion systems in heterologous common laboratory strain is highly desirable. Furthermore, it will be convenient to produce these components of TPS systems at large scale in nonpathogenic bacteria. In the current study we thus produced recombinant TPS systems of *N. meningitidis* in a well-known laboratory strain of *E. coli* to facilitate convenient analysis of the systems.

Recombinant protein production in heterologous host can be tricky in some cases, particularly if host species is distantly related to the organism from which the gene was cloned. In such cases most of the time strategies like optimized plasmid, promoter and expression level would need to be worked out. In some cases, it even requires to sort out with rare codons and replace it with frequently used codons while keeping the encoded protein sequence conserved. In our case, however, *E. coli* and *N. meningitidis* are evolutionary related species, and as expected, codon optimization was not required. However, there have been examples where proteins from *N. meningitis* were proven toxic that resulted into the lysis and killing of the host cells and was thus was not possible to express those proteins (Barlow *et al.*, 1987, 1988). As a result of recombinant proteins production and gene expression in heterologous host, useful information have been obtained including elucidation of vital metabolic pathways and identification of immunogenic proteins for vaccine production (Mustafa *et al.*, 2013; Chen *et al.*, 2017; Cai *et al.*, 2018).

In some cases, particularly, in the case of outer membrane proteins such as autotransporters, it has been reported that replacement of a C-terminal motif, which was highly conserved among the neisserial outer membrane proteins, with the signature of the *E. coli* protein resulted into better localization, integration and ultimately physiological function (Robert *et al.*, 2006). However, in the case of TpsB transporter, we did not experience problems requiring such a replacement most probably due to the less conserved C-terminal motifs in the TpsB transporters. Furthermore, induction intensity and interval are also crucial factors determining successful expression of proteins in heterologous host. In our case, different IPTG evels for various time points have been added to determine the optimal harvesting point (Fig. 2). Our results indicated that 100 m*M* IPTG for 30 min induction time produced an overall better TPS proteins and optimal secretion in the medium. Interestingly, however, in the wild type *N. meningitidis* host, TPS-2 system had better expression and secretion pattern when induced at least for 3 h (Sadeeq-ur-Rahman *et al.*, 2014). This is more probably due to the nature of promoter (lac promoter in this case) and neisserial expression vector (pEN plasmid) that has relatively lower expression rate as compared to inducible T7 promoters. Overall, our study indicates that it is convenient to express and analyze TPS systems from *N. meningitidis* in *E. coli* laboratory strains. Further work should be focused to express and optimized secretion of other ortholog TPS systems of *N. meningitis* in *E. coli* and production of TpsB and TpsA fragments to obtain crystals in complex form that would ultimately tell us the interaction interface of these two interacting partners.

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