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Expression and analysis of two-partner Secretion system of *Neisseria meningitidis* using heterologous host *Escherichia coli*

Sadeeq ur Rahman [1]*, Attaur Rahman[1], Ijaz Ali[4], Hamayun Khan[5], Muhammad Akbar Shahid[6], Nazir Ahmed Khan[2] and Irshad Ahmed[3]

[1]Abdul Wali Khan University, College of veterinary Sciences and AH, Garden Campus, Mardan, Khyber Pakhtunkhwa-Pakistan
[2] Deparment of Animal Nutrition, The University of Agriculture Peshawar Pakistan
[3]Astbury Centre for Structural Molecular Biology and School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, United Kingdom
[4] Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan
[5] Deparment of Animal Health, The University of Agriculture Peshawar Pakistan
[6] University of veterinary and animal scinces Lahore Pakistan
*) corresponding author
e-mail: Dr. Sadeeq ur Rahman, E-mail: Sadeeq@awkum.edu.pk tel.: +92-937-9230657-58 Ext; 1057

Abstract: In Two-Partner Secretion (TPS) systems of Gram-negative bacteria, the outer membrane-embedded TpsB transporter is dedicated for the transport of a large exoprotein, generically called TpsA. TpsBs consist of a C-terminal 16-stranded β barrel and two periplasmic polypeptide transport- associated (POTRA) domains that initiate outer membrane translocation once engaged with the N-terminal two-partner secretion (TPS) domain of TpsA protein. *Neisseria meningitidis* (NM), a commensel of human oral-mucosal epithelium that can sometime leads to sepsis and meningitis, encodes complex sets of TPS associated open reading frames comprising a total of two TpsB-transporters and five TpsA secreted proteins. Mechanism of secretion of TpsA protein and molecular determinants of interaction interface are still to understand. Expression and optimization of neisserial TPS systems in ordinary laboratory host strains such as *E. coli* MC1061 is highly desirable for convenient handling and reduced hazards associated with pathogens. Therefore, in the current study, we reconstituted TPS-2 of NM by cloning TpsB2 downstream to its substrate TpsA protein in neisserial expression vector pEN and expressed them in heterologous host *Escherichia coli* MC106 and its orthologs DHB4. Our results indicate that TPS system 2 secretes its TpsA substrate as efficient as like in the wild type host NM. The secretion was 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 NM. The tool will be useful to further investigate and unravel the molecular determinants involved during initial interaction and most importantly for large scale production of the recombinant TPS components for downstream procedures such as crystallography and protein localization studies.

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Key words: Two-partner secretion systems, heterologous protein expression, Neisseria meningitidis, TpsA , TpsB

Introduction

The Two-Partner Secretion (TPS) systems of Gram-negative bacteria are a distinct subclass of the Type V secretion pathway, which also includes the classical and trimeric autotransporters (Grijpstra *et al.*, van Ulsen, Rahman, Daleke, *et al.*). TPS systems consist of minimally two proteins; a secreted TpsA protein and a dedicated TpsB transporter that is embedded in the outer membrane. TpsA proteins are typically over 100 kDa and have a predominantly -helical structure (Kajava *et al.*, Kajava & Steven). They have been reported to function as adhesins, toxins with bacterial or eukaryotic targets or receptors/scavengers of nutrients from the environment (van Ulsen, Rahman, Daleke, *et al.*, 2014). The TpsB transporter secretes the TpsA protein across the outer membrane in a process that is not fully understood. Both TpsA and TpsB are produced with an N-terminal signal peptide and are transported across the inner membrane to the periplasmic space via the Sec translocon. TpsB subsequently inserts in the outer membrane to facilitate the secretion of the TpsA. The TpsA N-terminal domain of ~250-300 residues

called the TPS domain is crucial for this interaction (Jacob-Dubuisson *et al.*).

The TpsB proteins belong to the Omp-85 super family of proteins that include the BamA protein involved in the biogenesis outer membrane-based β-barrel proteins and its eukaryotic homologs (Jacob-Dubuisson *et al.*, 2009). The family is typified by a C-terminal 16-stranded β-barrel preceded by a soluble module that includes one to five polypeptide transport-associated (POTRA) domains (two in the case of TpsB). The crystal structure of FhaC, the TpsB transporter of filamentous haemagglutinin of *B. pertussis* (FHA), corroborated this overall organization (Clantin *et al.*, 2007). A particular feature of FhaC and other TpsBs is that the interior of the FhaC β-barrel domain is occupied by an α-helix that precedes the two POTRA domains in the sequence and an extended external loop (loop 6) that folds inward. Despite a high sequence diversity, the POTRA domains of the Omp85-family members adopt a similar βααββ configuration that folds into a three-stranded β-sheet overlaid with two anti-parralel α-helices, although the α_2 is missing in POTRA1 of FhaC (Clantin *et al.*, 2007) (Kim *et al.*, 2007) (Gatzeva-Topalova *et al.*, 2008) (Koenig *et al.*, 2010) (Arnold *et al.*, 2010). POTRA domains interact with the target proteins of their transporter (Habib *et al.*, 2007) (Kim *et al.*, 2007) (Knowles *et al.*, 2008) and it has been shown that POTRA domains bind 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).

The Gram-negative diplococcus *Neisseria meningitidis* (meningococcus) is a major cause of meningitis and sepsis world-wide (Stephens *et al.*, 2007). *N. meningitidis* genomes encode up to three TPS systems and the system has been implicated in pathogenesis (van Ulsen *et al.*, 2008b, van Ulsen & Tommassen, 2006). In brief, system 1 and 2 comprised of two TpsAs and one TpsB each while system 3 contain an orphan TpsA protein. We recently showed that TpsB1 of system showed substrate specificity, whereas TpsB2 was shown to secrete TpsAs of other TPS systems as well (Rahman & van Ulsen, ur Rahman, Arenas, Tommassen, *et al.*). We have shown previously that system specificity is conferred by the periplasmic POTRA domains (ur Rahman, Arenas, *et al.*). Sequence alignments of TpsB proteins indicate high diversity within the POTRA region; however, there is quite high degree of similarity in the barrel region, while some conserved motifs have been mapped. TPS domain, the N terminal ~250 residues that contain signal for secretion, of TpsA protein interacts first with the POTRA domains, however, it is not known exactly which particular residues and regions are crucial for this initial engagement. Similarly, very little is known about the interaction interfaces of TPS domain with the POTRA domain or elsewhere of TpsB proteins. To unravel the molecular mechanism of this early and the following sequels of interactions a convenient approach for production of these proteins that is reproducible at various scales and easily accessible and dissectible in non pathogenic recombinant host would be highly desirable. This expression system that can be analsysed in ordinary lab would be ideal to reconstitute the TPS system of NM, pathogenic strain that needs much care during work, in non pathogenic *E. coli* laboratory strain. Here, we report on such a heterologous system based on non pathogenic *E. coli* strain that can be conveniently used for analysis of TPS system of NM.

Materials and methods

Bacterial strains and growth conditions

The *N. meningitidis* (NM) and *E. coli* strains used for this study are listed in Table 1. *N. meningitidis* strains were grown on GC agar (Oxoid) supplemented with Vitox (Oxoid) at 37°C, 5% CO_2. Liquid cultures of *N. meningitidis* strains were grown at 37°C in tryptic soy broth (Gibco-BRL). Chloramphenicol was added to a final concentration of 8 μg/ml for plasmid selection. *E. coli* strains were grown on lysogeny broth (LB) or grown on LB agar plates supplemented with 100 μg/ml ampicillin or 30 μg/ml chloramphenicol for plasmid maintenance and with 0.5% glucose for full repression of the *lac* operator, when appropriate.

Most of the work was performed at molecular microbiology lab of VU university Amsterdam, The Netherlands, while, a part was performed at Animal health department of agriculture university Peshawar Pakistan.

Cloning and construction

TpsB2 gene was amplified by PCR using a set of primers (pr_tpsB2_F:CACATATGGATCCGTGTATTGAATGCCCATTGATGA,pr_tpsB2_End: GAGATCTGAATTCCGTTGATTTGACTATGCCGTTTTA)and high fidelity DNA polyermerease (Roche) from chromosal DNA of HB-1 *N. meningitidis* and the resulted amplicon was cloned into pGEMT ((Promega) (Table 2) (ur Rahman, Arenas, *et al.*) that was then confirmed by sequencing (Macrogen) (van Ulsen *et al.*). The ORF t*psB2* was combined with a truncated *tpsA2* ORF (Rahman & van Ulsen), the ORF was excised from pET11a using BamHI/EcoRI restriction sites. The combined ORFs were then cloned from the resulting plasmid into the pEN expression vector using NdeI/AatII (see Fig 1).

SDS-PAGE and Western blotting

All procedures were carried out as described earlier [22, 25]. Briefly, *N. meningitidis* HB-1 cultures were grown for 4-5h to an OD_{600} of ~2.5.0-3.5, in the

presence of 1- to 0.1mM of isopropyl-beta-d-thiogalactopyranoside (IPTG) where indicated. Cells were harvested by centrifugation (4,500× *g*, 5 min) and the pellet was resuspended in phosphate-buffered saline pH 7.4 (PBS) to a final OD_{600} of 10. Culture supernatants were centrifuged (16,000×g, 10 min) to remove residual cells. Occasionally the culture supernatant was further subjected to ultracentrifuge (200,000×g) in a bench top ultracentrifuge (Beckman and Coulter). 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). Cultures of *E. coli* strains containing pEN plasmids were grown in LB broth to an OD_{600} of ~0.4-0.6. IPTG was added to a final concentration of 0.1- to 1- mM and incubation was resumed for another 1-2 hrs. 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 (Rahman & van Ulsen) and incubated with sera diluted 1:5,000 (anti-TPS1 and anti-TPS2a) or 1:10,000 (anti-TpsB1 and anti-TpsB2), 1:10,000 (anti-DegP). The secondary antibody used was goat anti-rabbit immunoglobulin G serum conjugated to horseradish peroxidase (Biosource International), diluted 1:10,000. The binding of antibodies to proteins on the blots was visualized using Lumilight- normal or Plus (Roche). The indicated relative molecular weight of the protein was deduced from the Precision Plus Protein Standard (BioRad) including in each SDS-PAGE gel.

Outer membrane isolation and heat modifiability

Outer membrane fractions were isolated according to (van Ulsen *et al.*). Cells were harvested by centrifugation (4,500× *g*, 5 min). The pellet was resuspended in a 50 mM Tris-HCl pH 8.0 and 2 mM EDTA buffer and were passed 2 times through a One Shot Cell disrupter (Constant Systems Ltd) at 30,000 psi. Unbroken cells were pelleted by centrifugating the lysate (4,500× *g*, 5 min). The supernatant was then subjected to ultracentrifugation (200,000×g, 30 min). The resulting pellet containing a crude outer membrane fraction was resuspended in PBS to represent a culture of OD_{600} ~20. The denatured samples were further diluted in 2×sample buffer and boiled for 10 min. The native samples 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. Samples were loaded on semi-native SDS-PAGE gels, that are prepared without SDS in the gel and run at 12 mA for at the least 3 h while cooled in ice. Blotting was performed as described above.

Results

Expression and secretion analysis of TPS-2 in wild type and heterologous *E. coli* hosts

To express and analyze TPS-2 of NM in non pathogenic laboratory strain such as *E. coli* MC1061, we cloned TpsB2 downstream to its truncated TpsA (TPS2a) substrate protein in neisserial pEN vector (Fig 1). The pEN plasmid has IPTG inducible *lac* promoter downstream to which TPS-2 system was cloned. The final construct (pEN1220) was then transformed to electro-competent *E. coli* MC1061 cells by heat shock method. Successful transformants were selected on chlormaphenicol containing LB agar plates. Of each independent clone, three colonies were further screened for expression and secretion analysis.

To express the TPS-2 system we added IPTG (100 mM) to the growing cells at log phase of the cell growth. We then collected the cells after two hrs 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. We then compare the expression and secretion of TPS2a in the wild type host NM 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 was comparable to wild type host.

Effect on growth pattern and cell viability

It is well known that expression of proteins of NM in *E. coli* is associated with toxicity, which is probably as a result of over expression or toxic effects of the recombinant protein of NM that ultimately cause cell lysis of the host strain. For this purpose we checked the growth pattern of wild type *E. coli* MC1061 expressing recombinant TPS-2 strain. Growth curve analyses indicated that cells were growing normal as like wild type without plasmid and uninduced cells indicating no detectable adverse effects (results not shown).

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. It is known that outer membrane proteins once integrated and properly folded; show a particular heat modifiability pattern, indicated by folded and faster specie that can be linearised by adding reducing agent such Dithiothreitol (DTT)(Ur Rahman, Arenas, *et al.*, 2014b). 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 NM (Fig. 2B). Similar results were also found in *E. coli* DHB4 (data not shown).

Optimization of secretion

We next then determine the optimal dose and duration of induction for optimal production. For this purpose, different amounts such as 0-, 50-, 100-, 200-, 500- mM of IPTG was added and cells were grown for maximum of 2 hrs after 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 mM 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 mM IPTG. Our results indicated that 2 hrs of total induction produced maximum proteins (Fig 2C). Overall, our results indicate that heterologous expression of neisserial TPS-2 systems in *E. coli* MC1061show the features of wild type and thus can be in fact used for secretion analysis and TPS characterization.

Discussion

TPS systems got a lot of attention in the recent years due to its conservation in pathogenic and non pathogenic Gram negative strains and its diverse role in the virulence. In addition to its rule in virulence such as biofilm formation, intracellular survival and escape from infected cells; killing effect of contact dependent growth inhibition revolutionized research about TPS systems (van Ulsen, Rahman, Jong, *et al.*, 2014). Remarkably, the transporter TpsB protein of the TPS systems belongs to Omp85-super family proteins that function as integrase or translocase; two of the cell`s vital and basic functions. Mechanism of outer membrane integration and translocation has not been fully understood and so far BamA and TpsB FhaC proteins have been used as modular proteins for these kinds of studies (Noel *et al.*). Based upon FhaC crystal structure, (Clantin *et al.*) TpsB model suggests that POTRA domains occupy region that remains exposed in the periplasm (van Ulsen, Rahman, Jong, *et al.*, 2014). There are conserved stretches of positive and negative charges, while the grooves are filled by mostly hydrophobic elements. 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. It will be convenient to produce these components of TPS systems at large scale in non pathogenic bacteria as optimized as like the wild type host NM. In the current study we thus produced recombinant TPS systems of NM in well know laboratory strain *E. coli* to facilitate convenient analysis of the systems.

Recombinant protein production in heterologous host can be tricky in some cases when the species are unrelated. In such cases most of the time strategies like optimized plasmid, promoter and expression level would need to work out, but depending on how distant the species related to the expression host. In some cases even it requires to sort out the rare codons and replace it with frequently used codons while keeping the protein resultant sequence conserved. However in other cases, the overall codon optimization is required because of the distant relationship of the source and host organisms. In our case, however, *E. coli* and *N. meningitidis* are evolutionary related species, and as expected, codon optimization would not have been 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 not possible to express those proteins (Barlow *et al.*, 1987, 1988). Although, based on prediction and physiological properties of those expressed proteins indicate that they are not normally toxic. In some cases, particularly, in the case of outer membrane proteins such as autotransporters, it has been reported that replacement of the last 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. However, in the case of TpsB transporter, we did not experience such a replacement most probably due to the less conserved C-terminal motifs in the TpsB transporters. Overall, our study indicates that it is convenient to express and analyse 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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Figures and legends

Fig. 1: A. TPS systems of *N. meningitidis* and its recombinant production in *E. coli*. (Top) Cartoons of chromosomal localization and distribution of TPS associated genes in the chromosome of NM. The size and position of the TPS associated genes are not drawn to scale and these represent approximate position and size. Dashed line below TpsA3 indicates the

approximate size of the signal sequence plus TPS domain that was cloned (bottom) cartoon representation of the vector containing genes of interest that encode TpsA and TpsB partners that was used in this study.

Fig. 2: Expression and optimization of TPS-2 systems of *N. meningitidis* in *E. coli* strain. **A**. Immunoblots of whole cell lysates of *N. meningitidis tpsB1::kan/tpsB2::gen* and *E. coli* cells carrying plasmids encoding pEN1220 that express TpsB2 and truncated TPS2a substrate as indicated. Samples were induced or not with IPTG as indicated above. **B.** Immunoblots of outer membranes isolated from induced cells containing plasmid pEN1220 that expresses TPS-2 system of N. meningitidis in its natural host and heterologous host E. coli as indicated above the lanes. Samples were incubated with or without DTT (**C&D**) immunoblot of whole cell lysates and culture supernatant expressing TPS-2 systems of N. meningitidis with different amounts of IPTG and duration of induction as indicated above the lanesThe 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) along side of the samples.

Table 1: Neisserial expression and cloning vectors used in this study

strains and constructs	Plasmid name	TPS ORFs	reference
***N. meningitidis* strains**			
HB-1 *tpsB1::kan tpsB2::gen*			(ur Rahman & van Ulsen, 2013)
			current study
***E. coli* strains**			
Top10F			Invitrogen
DHB4			(Brandon & Goldberg, 2001)
MC1061			(Casadaban & Cohen, 1980)
MC1061:: *degP*::*S210A*			(Spiess *et al.*, 1999)
Plasmids			
TPS2a+TpsB2	pEN1220	*tpsA2a*-tr + *tpsB2*	(ur Rahman & van Ulsen, 2013)
TPS2a	pPU1200	*tpsA2a*-tr	
Cloning vectors			
	pET11a		Novagen
	pGEM-T		Promega
	pPU1000		(van Ulsen *et al.*, 2008a)
	pPU1200		
	pEN		

[a] The suffix "–tr" indicates that the construct comprises a truncated *tpsA* ORF that encodes the signal peptide and the TPS domain (Rahman & van Ulsen).

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9/21/2022