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Functional Analysis of the Twin-Arginine Translocation Pathway in *Sodalis glossinidius*, a Bacterial Symbiont of the Tsetse Fly[▽]

Linda De Vooght,[1] Guy Caljon,[2,3,4] Marc Coosemans,[1] and Jan Van Den Abbeele[2]*

Department of Parasitology, Unit of Entomology, Institute of Tropical Medicine Antwerp (ITM), Antwerp, Belgium[1]; Department of Animal Health, Unit of Veterinary Protozoology, Institute of Tropical Medicine Antwerp (ITM), Antwerp, Belgium[2]; Unit of Cellular and Molecular Immunology, Vrije Universiteit Brussel (VUB), Brussels, Belgium[3]; and Department of Molecular and Cellular Interactions, VIB, Ghent, Belgium[4]

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This study demonstrates a functional twin-arginine (Tat) translocation pathway present in the tsetse fly symbiont *Sodalis glossinidius* and its potential to export active heterologous proteins to the periplasm. Functionality was demonstrated using green fluorescent protein (GFP) fused to the Tat signal peptide of *Escherichia coli* trimethylamine *N*-oxide reductase (TorA).

Expression of proteins that interfere with pathogen development by genetically modified symbiotic bacteria of arthropod disease vectors may serve as a powerful approach to control disease transmission (paratransgenesis). The use of bacterial symbionts expressing foreign proteins in disease-carrying arthropods has also an intriguing potential for studying insect-pathogen interactions (2). *Sodalis glossinidius*, a maternally inherited Gram-negative symbiont of the tsetse fly, is one of the few insect symbiotic bacteria that can be cultured and genetically modified *in vitro* (2, 6, 19) and is currently considered a promising drug delivery tool to block trypanosome development in the tsetse fly (1). Active protein secretion is crucial to target the effector molecules to where the trypanosome parasites reside. To date, there have been no studies regarding the export of heterologous proteins to the periplasmatic and/or outer environment of *S. glossinidius*.

In prokaryotes, most secreted proteins are translocated across the cytoplasmic membrane in an unfolded conformation by the Sec pathway (for a comprehensive review, see reference 10). A recently described Sec-independent pathway mediates the export of proteins in a folded conformation. This alternative pathway has been designated the twin-arginine translocation (Tat) system because of the characteristic twin-arginine motif (S/T-R-R-x-F-L-K) present in the signal peptide of proteins translocated via this system (3, 11, 13, 18, 20; for a recent comprehensive review, see reference 9). In *Escherichia coli*, a functional Tat pathway requires a minimum set of three gene products: TatA, TatB, and TatC (4). In the present study, we report on the presence of a functional Tat secretory pathway in *S. glossinidius* and its potential to be exploited for export of heterologous proteins in an active manner to the periplasm.

Analysis of the completed *S. glossinidius* genome (GenBank accession no. NC_007712) (16) revealed the presence of a *tat* locus, composed of *tatA*, *tatB*, and *tatC* (GenBank accession no., respectively, SG0112, SG0113, and SG0114), on the circular chromosome with a high level of homology to the corresponding *E. coli* genes and with identities ranging from 47 to 77% on the amino acid level. A putative promoter was identified (BPROM Server algorithm) 421 bp upstream of *tatA*, while the close proximity of the individual *tat* genes (4 bp between *tatA* and *tatB* and 4 bp between *tatB* and *tatC*) is suggestive for an arrangement as a single operon. Topology prediction (TMpred; http://www.expasy.org/) suggested similar membrane insertion of the *S. glossinidius* TatABC proteins compared to the corresponding *E. coli* counterparts. The extent to which this secretory pathway is used in prokaryotes strongly

TABLE 1. List of predicted Tat-dependent proteins of *Sodalis glossinidius* with their predicted subcellular localizations and biological functions

Sodalis glossindius protein	Accession no.	Localization	Biological function
N-Acetylmuramoyl-L-alanine amidase (AmiC)	YP_455641.1	Periplasm	Cell envelope biogenesis
Proline aminopeptidase P II (PepP)	YP_455685.1	Cytoplasm	Amino acid transport and metabolism
Membrane protein TonB (TonB)	YP_455061.1	Outer membrane	Iron ion transmembrane transporter activity
Cell division inhibitor (MinD)	YP_455015.1	Cytoplasm	Cell cycle
Cell division protein (FtsN)	YP_455847.1	Inner membrane	Cell cycle
3-Oxoacyl-(acyl-carrier-protein) reductase	YP_454740.1	Cytoplasm	Oxidoreductase

* Corresponding author. Mailing address: Department of Animal Health, Unit of Veterinary Protozoology, Institute of Tropical Medicine Antwerp (ITM), Nationalestraat 155, B-2000 Antwerp, Belgium. Phone: 32.3.2476262. Fax: 32.3.2476359. E-mail: jvdabbeele@itg.be.

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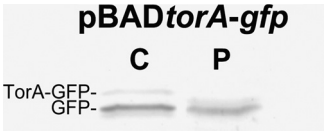

FIG. 1. Expression and export of TorA-GFP by the Tat pathway in *Sodalis glossinidius* cells. Expression was induced with arabinose. The location of the expressed GFP was analyzed by immunoblotting of periplasmic (P) and cytoplasmic (C) fractions using an anti-GFP antibody (1:3,000; Sigma) for detection. Presented data are representative for two independent experiments.

varies according to bacterial species and is not yet well characterized (8). A genome survey to identify putative Tat substrates in the predicted proteome of *Sodalis glossinidius* using "pattern search" in the Pedant database (http://pedant.gsf.de) resulted in the identification of six substrates (Table 1). The search pattern used was x-RR-x-[VFLIMA]-x. The results from the pattern search were analyzed using the TatP program (http://www.cbs.dtu.dk/services/TatP). To reduce the number of false positives, we excluded all proteins that had negative results for two or more parameters measured by the program. The majority of these predicted Tat substrates require cofactor binding and appear primarily involved in redox regulation, which are recurrent features of Tat substrates in other prokaryotes.

For investigation of Tat functionality in *S. glossinidius*, the RR-signal peptide of TMAO reductase (TorA), a molybdopterin-containing protein that is known to be exported by the Tat pathway in *E. coli* (11, 13), was used. The TorA signal sequence was demonstrated to be highly Tat specific and has been efficiently employed to target heterologous proteins to the periplasm of various prokaryotes (5, 7, 17). The pBAD*torA-gfp* plasmid (15), encoding a green fluorescent protein (GFP) reporter fused to the *E. coli* TorA signal peptide, was introduced into *S. glossinidius* via a heat shock procedure (2). Cells were grown to mid-exponential growth phase (optical density at 600 nm [OD_{600}] = 0.2) before TorA-GFP expression was induced by arabinose (0.15%) for 5 h. Next, the location of the expressed GFP was monitored by immunoblotting of periplasmic and cytoplasmic cell fractions. Cells were fractionated according to the method of Skerra and Plückthun (14). Samples were heat denatured at 95°C in the presence of SDS-PAGE loading buffer and analyzed on a 12% (wt/vol) polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Whatman) and blocked overnight in 5% skim milk to be assayed by Western blot analysis using an anti-GFP detection antibody (1:3,000; Sigma). Figure 1 shows that two forms of GFP could be detected in *S. glossinidius* cells harboring the pBAD*torA-gfp* plasmid: precursor GFP (30 kDa) and a mature-sized protein with a molecular mass corresponding to that of GFP devoid of the TorA leader sequence (27 kDa). Cytoplasmic extracts contained both mature and precursor forms, while only the mature-sized GFP could be detected in the periplasm, indicating the predicted cleavage of the TorA leader sequence during periplasmic transport.

Kinetics and activity of the periplasmically transported GFP were assessed by fluorimetry on prepared cell fractions (Fig. 2). *S. glossinidius* cells expressing TorA-GFP were induced at 26°C for 2 h using 0.15% arabinose. To prevent saturation of the Tat pathway, cells were washed to remove the arabinose from the culture medium and subsequently 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added to repress the pBAD promoter. Samples were taken immediately (t = 0), 2 h and 5 h after removal of arabinose. The relative fluorescence levels present in the different cellular fractions were quantified using a PerkinElmer Victor3 1420 multilabel counter (485-nm excitation/535-nm emission; measurement time, 0.1 s). At t = 0 and t = 2 h after arabinose removal, the majority of GFP was still located in the cytoplasm. However, 5 h after arabinose induction, a clear shift in the presence of fluorescence was observed from the cytoplasm toward the periplasm. We calculated that a maximal export efficiency of around 65% could be achieved within 5 h after induction, which is consistent with previous studies in *E. coli* (11). In *S. glossinidius* harboring the pBAD*gfp* control plasmid devoid of the TorA leader sequence, GFP clearly remained accumulated in the cytoplasm whereas only a small and constant amount of GFP could be detected in the periplasmic extracts.

The *in vivo* cellular localization of GFP in TorA-GFP-expressing cells was assessed more directly by fluorescence mi-

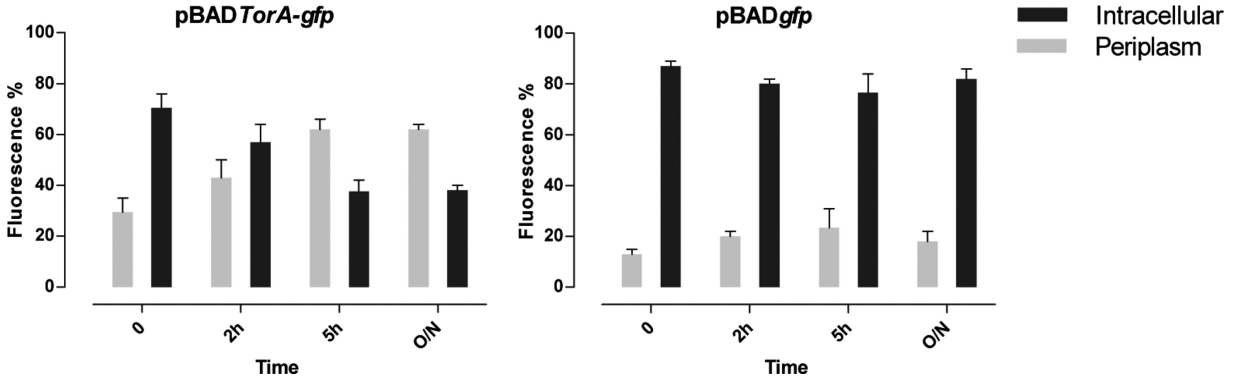

FIG. 2. Quantitative export of active TorA-GFP by the Tat-pathway in *Sodalis glossinidius* cells. Cells were grown in the presence of arabinose, and samples were taken immediately (0), 2 h, 5 h, and 24 h (O/N) after removal of arabinose and IPTG repression. Values are given for the fluorescence of periplasmic and cytoplasmic fractions of cells harboring the pBAD*torA-gfp* and pBAD*gfp* plasmid. As a control to determine the background fluorescence, cell fractions from noninduced *S. glossinidius* containing the respective plasmids were used. Presented data are representative for three independent experiments; data in the graph are means ± standard deviations.

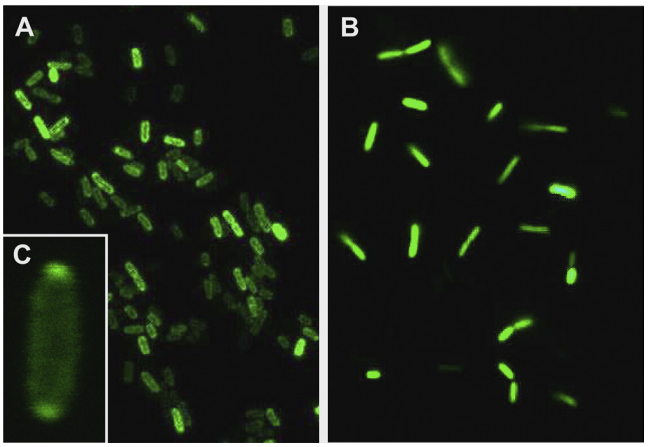

FIG. 3. Localization of GFP in cells harboring the pBAD*torA-gfp* (A) and pBAD*gfp* (B) plasmid by fluorescence microscopy. Samples were immobilized on poly-L-lysine-coated slides and analyzed on an Olympus BX-41 UV microscope equipped with a filter set for fluorescein isothiocyanate and a 60× (U Plan FLN 1.25) oil immersion objective lens. Occasionally, GFP was found to accumulate at the poles of pBAD*torA-gfp*-containing cells (C). Presented data are representative for at least three independent observations.

croscopy (Fig. 3). In concordance with the fluorimetry experiment, cells were induced by arabinose for 2 h and samples were taken immediately, 2 h, and 5 h after washing and repression with IPTG. Consistent with the fluorimetry data, immediately and 2 h after arabinose removal, GFP remained uniformly distributed in TorA-GFP-expressing cells. However, 3 h later ($t = 5$ h), cells showed prominent halos of periplasmic GFP. Occasionally, GFP was observed to concentrate at the poles in the periplasm. This polar compartmentalization has been shown to be reversible and is suggestive of free movement of proteins within the periplasm in response to environmental changes (12). In *S. glossinidius* cells harboring the pBAD*gfp* control plasmid, GFP remained uniformly distributed, with no signs of enrichment of the signal in the periplasm. Collectively, these data confirm that active GFP specifically accumulates in the periplasm, which results from the recognition of the TorA signal peptide that is cleaved off upon transport.

So far, the functionality of the bacterial Tat system has been analyzed mainly in free-living organisms. In this study we demonstrated that the Tat pathway is biologically active in *Sodalis glossinidius*, a bacterial symbiont of the tsetse fly, and identified a number of predicted natural endogenous substrates that contain the characteristic twin-arginine motif. Moreover, we illustrated that the Tat system has the potential to be exploited for exporting heterologous proteins in an active manner to the periplasm, which will be further explored for paratransgenesis approaches to control trypanosome infection in the tsetse fly vector.

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