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RESEARCH PAPER/REPORT



## Characterization of recombinant *Trypanosoma brucei gambiense* Translationally Controlled Tumor Protein (r*Tbg*TCTP) and its interaction with *Glossina* midgut bacteria

Géraldine Bossard<sup>a</sup>, Manon Bartoli<sup>b</sup>, Marie-Laure Fardeau<sup>b</sup>, Philippe Holzmüller<sup>c</sup>, Bernard Ollivier<sup>b</sup>, and Anne Geiger<sup>d</sup>

<sup>a</sup>CIRAD, UMR INTERTRYP, Montpellier, France; <sup>b</sup>IRD-MIO, Marseille, France; <sup>c</sup>CIRAD, UMR CMAEE (control des maladies animales exotiques et émergentes), Montpellier, France; <sup>d</sup>IRD, UMR INTERTRYP, Montpellier, France

### ABSTRACT

In humans, sleeping sickness (*i.e.* Human African Trypanosomiasis) is caused by the protozoan parasites *Trypanosoma brucei gambiense* (*Tbg*) in West and Central Africa, and *T. b. rhodesiense* in East Africa. We previously showed *in vitro* that *Tbg* is able to excrete/secret a large number of proteins, including Translationally Controlled Tumor Protein (TCTP). Moreover, the *tctp* gene was described previously to be expressed in *Tbg*-infected flies. Aside from its involvement in diverse cellular processes, we have investigated a possible alternative role within the interactions occurring between the trypanosome parasite, its tsetse fly vector, and the associated midgut bacteria. In this context, the *Tbg tctp* gene was synthesized and cloned into the baculovirus vector pAcGHLT-A, and the corresponding protein was produced using the baculovirus *Spodoptera frugicola* (strain 9) / insect cell system. The purified recombinant protein r*Tbg*TCTP was incubated together with bacteria isolated from the gut of tsetse flies, and was shown to bind to 24 out of the 39 tested bacteria strains belonging to several genera. Furthermore, it was shown to affect the growth of the majority of these bacteria, especially when cultivated under microaerobiosis and anaerobiosis. Finally, we discuss the potential for TCTP to modulate the fly microbiome composition toward favoring trypanosome survival.

### ARTICLE HISTORY

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### KEYWORDS

bacteria midgut flora; *Glossina*; *Trypanosoma brucei gambiense*; Translationally Controlled Tumor Protein (TCTP)

## Introduction

*Trypanosoma brucei gambiense* (*Tbg*) and *T. b. rhodesiense* are protozoan parasites that cause sleeping sickness (Human African Trypanosomiasis/HAT), respectively in West and Central Africa, and in East Africa. It is a neglected tropical disease endemic to many sub-Saharan African countries and responsible for severe social and economic issues. Several biologic control strategies have been developed over the years, including the control of tsetse fly populations by aerial insecticide applications<sup>1–3</sup> and/or by the sterile insect technique.<sup>4</sup> Moreover, various diagnostic tools have been developed and improved,<sup>5–12</sup> as well as the treatments of infected humans.<sup>13</sup> However, the disease still has a strong presence and continuing research is required to fight it.

Sleeping sickness is propagated by a hematophagous vector, the tsetse fly, which ingests the trypanosome parasite during a blood meal on an infected host. The trypanosome must then fulfill part of its complex biologic

cycle in the fly before it is transmitted to yet another mammalian host when the fly consumes a subsequent blood meal. As a HAT control strategy, interrupting a step within the trypanosome life cycle inside the fly could become an effective approach to prevent its transmission. In this context, several studies have already investigated the molecular dialog occurring between the tsetse fly, its midgut bacteria and the ingested trypanosomes to identify factors involved in these interactions that could become potential targets to hinder vector competence.

Previous investigations have allowed identifying several proteins that are excreted/secreted by trypanosomes.<sup>14</sup> A high number of midgut proteins were also identified as differentially expressed in *Tbg*-stimulated versus non-stimulated *Glossina palpalis gambiensis* flies.<sup>15</sup> Similarly, numerous tsetse, midgut bacteria and trypanosome genes were found to be differentially expressed in *Tbg*-infected vs. non-infected tsetse

flies.<sup>16</sup> Among these proteins we identified the trypanosomal Translationally Controlled Tumor Protein (TCTP), for which the corresponding gene was found to be overexpressed in *Tbg*-infected flies.

TCTP is a ubiquitously distributed protein in eukaryotes that appears to be highly conserved across animals and plants.<sup>17</sup> It has been reported to play multiple roles in cellular processes including cell proliferation,<sup>18,19</sup> the cell cycle,<sup>20</sup> stress response<sup>21</sup> and apoptosis,<sup>22</sup> and is involved in embryo development.<sup>23</sup> As such, TCTP can be considered a good therapeutic target against several diseases or physiological disorders.<sup>24-26</sup> Regarding the *Tbg* form of TCTP (*Tbg*TCTP), the fact that the protein can be excreted/secreted by the trypanosome raises a question about its paracrine role, since the parasite develops in the fly gut where several bacteria are harbored, some of which have been shown to be involved in tsetse fly vector competence.<sup>27,28</sup>

Here we report on the production of the *Tbg*TCTP recombinant protein (*rTbg*TCTP) and its biological effect on several bacterial strains that were previously

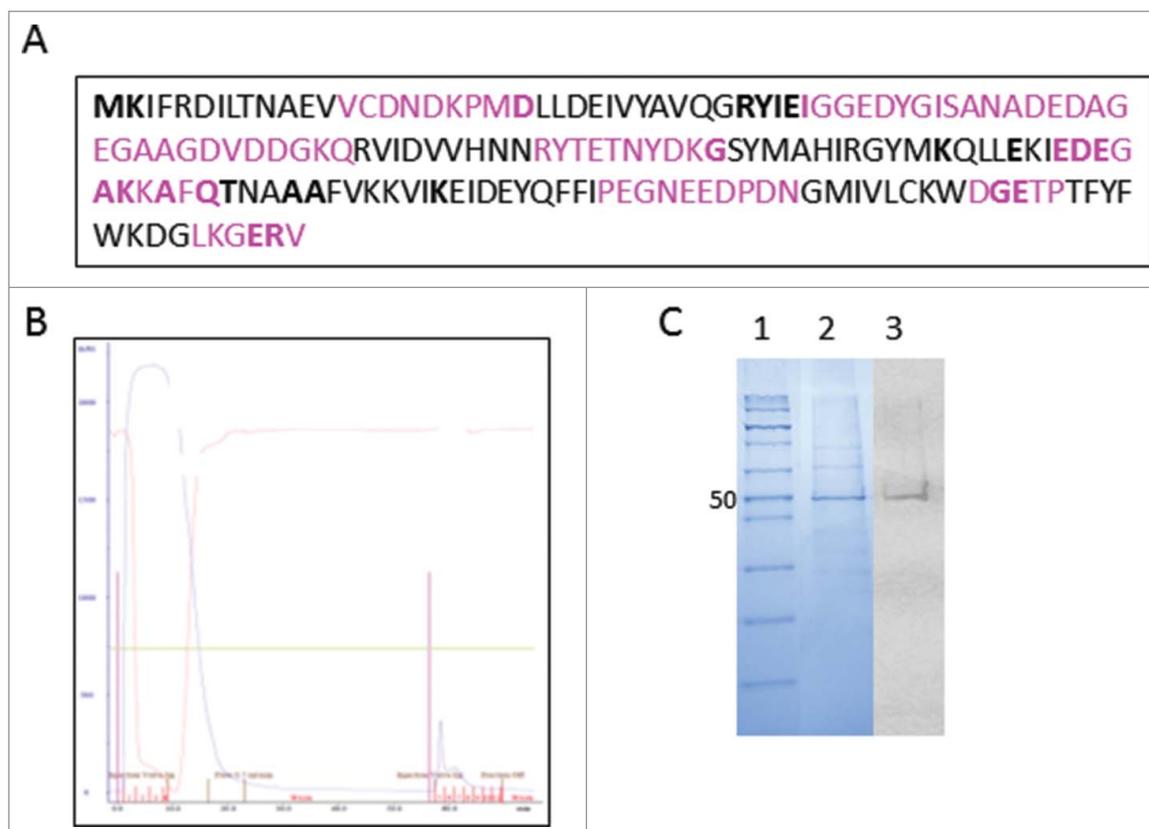
isolated from the midgut of diverse *Glossina* species sampled in different HAT foci.<sup>29-31</sup> These results will provide a basis for future research into controlling HAT from the perspective of the trypanosome parasite.

## Results

### Production, purification and molecular characterization of *rTbg*TCTP

The sequence of the synthesized *Tbg* 927.8.6750 gene exhibited a 100% match with the mRNA sequence from the NCBI database (AAX80036.1, EMBL-EBI). In addition, the amino acid sequence of the corresponding protein produced using the baculovirus *Spodoptera frugiperda* (Sf 9) / insect cell system displayed a 100% match with the TCTP amino acid sequence from the *T. brucei gambiense* reference strain TREU97 (Fig. 1A).

Figure 1B shows the result from purifying the produced *rTbg*TCTP on the Ni-NTA (nickel-charged affinity resin) agarose column. The purification process was continuously monitored (OD = 280 nm) and the first



**Figure 1.** (A) Protein sequence of *rTbg*TCTP with linearized epitope (pink); amino acids involved in the epitope conformation are presented in bold type. (B) Purification profile for *rTbg*TCTP on a nickel-charged affinity resin (Ni-NTA) column. (C) sodium dodecyl sulfate - PAGE analysis of *rTbg*TCTP. Lane 1: protein molecular marker (Novex Sharp Pre-Stained Protein Standard, Thermo Fisher Scientific); lane 2: electrophoresis of *rTbg*TCTP (10  $\mu$ g) purified on a Ni-NTA column stained with Coomassie Blue; lane 3: western blot of *rTbg*TCTP (10  $\mu$ g) on nitrocellulose membrane using anti-His monoclonal antibody (mAb).

peak of the chromatogram (representing proteins unbound to the Ni-NTA column) corresponded to non-specific proteins produced by the insect cells; the second peak eluted from the column contained the *rTbg*TCTP. The purified fraction was then loaded onto 10% SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) gels; after electrophoresis and staining, the presence of an intense protein band was shown at 50 KDa, corresponding to the molecular weight of *rTbg*TCTP (Fig. 1C, lane 2). The presence of *rTbg*TCTP in the 50 KDa protein band was further confirmed by immunodetection using HRP (horseradish peroxidase) - conjugated anti-histidine antibody (Fig. 1C, lane 3). The *rTbg*TCTP yield was approximately 174 mg per liter of insect cell culture medium, with a 95% purity rate (Bio 1D software).

### Protein structure and sequence homologies with TCTP from other organisms

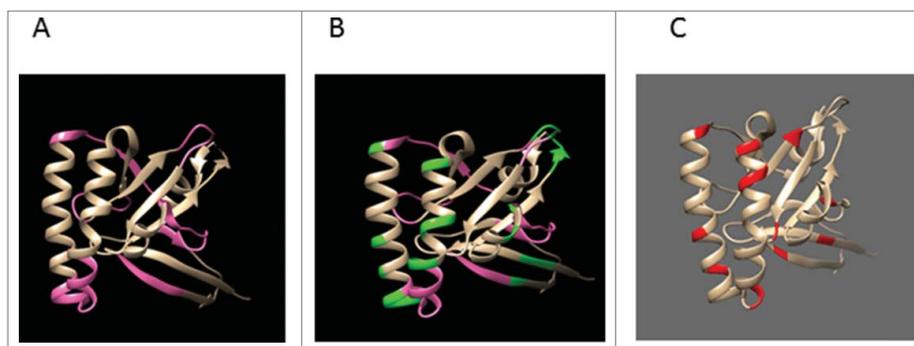
For our 3D structure analysis of TCTP, only a limited number of TCTP sequences were available in the EMBL-EBI database (Protein Data Bank in Europe) of 3D protein structures. Our analysis indicates that the *T. b. gambiense* *rTbg*TCTP amino acid sequence had an overall 35.5% homology with the TCTP model from *Plasmodium falciparum* (3P3K), although it displays homologous portions distributed homogeneously all along the protein sequence (Fig. 2A–C). The portions of sequences forming TCTP epitopes represent 46% of the total protein sequence; when the protein is linearized (*i.e.*, the primary structure), these portions are distributed homogeneously all along the protein sequence (Fig. 1A and Fig. 2A). Our results

also show that TCTP contains very few conformational epitope sites (Fig. 2B) or lysine sites (Fig. 2C).

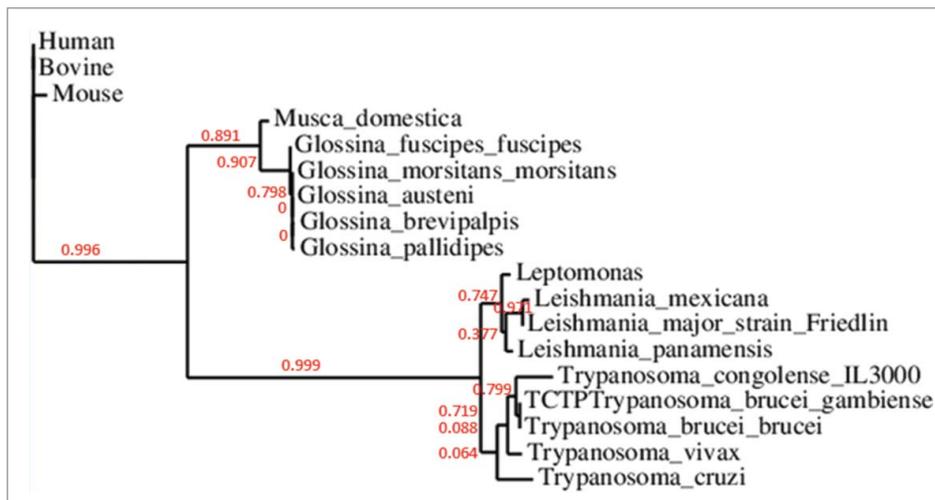
Figure 3 depicts a clustering of TCTPs from different species, based on differences in their respective amino acid sequences. The proteins from trypanosomatids cluster in a group that is clearly separate from that formed by the TCTP of different *Glossina* species, with 5 TCTPs from this latter group appearing almost identical. The genetic distance between this group and *Musca domestica* TCTP (used as a control) is shorter than the distance between TCTPs from flies and trypanosomatids. The TCTPs from trypanosomatids appear to be closely related (high homologies), even though those from *Trypanosoma* and *Leishmania* cluster into 2 distinct subgroups.

### Evidence of *rTbg*TCTP binding to *Glossina* midgut bacteria

The occurrence of binding between *rTbg*TCTP and *Glossina* midgut bacteria was revealed by western blot analysis. Figure 4 presents a representative result of the binding capacity between *rTbg*TCTP and *Providencia* bacteria species from *Glossina palpalis* midguts. As shown in Figure 4, an *rTbg*TCTP band (lane 4) similar to the *rTbg*TCTP control (lane 2) was only revealed when bacteria were incubated in the presence of *rTbg*TCTP, even though the bacteria were washed 4 times with PBS before being boiled and treated with  $\beta$ -mercaptoethanol. The fact that the PBS washing did not eliminate the *rTbg*TCTP indicates that the protein was bound to the bacteria wall, possibly through the formation of disulfide bonds between –SH groups of *rTbg*TCTP and the bacteria. No band was detected when *rTbg*TCTP were absent during bacteria incubation (lane 3). Among the 39



**Figure 2.** (A) The Tbg 927.8.6750 tertiary structure. The part corresponding to the epitope is in pink. (B) Linear epitope (pink) of Tbg 927.8.6750 from *Trypanosoma brucei gambiense* (Bepiprep 1.0 server) and the conformational epitope 3P3K (green; DiscoTope 2.0 server). (C) Lysine localization of 3P3K (red).



**Figure 3.** Phylogenetic tree for TCTP among trypanosomatids and their corresponding host species (PhyML). The PhyML website was used to construct the phylogenetic tree based on the amino acid sequences of the corresponding TCTP and default parameters of the server. Numbers above branches indicate bootstrap values.

bacteria species/strains that we examined (Table 1) for their capacity to bind *rTbgTCTP*, only 24 showed a positive result, whereas the others were unable to bind *rTbgTCTP* in our experimental conditions.

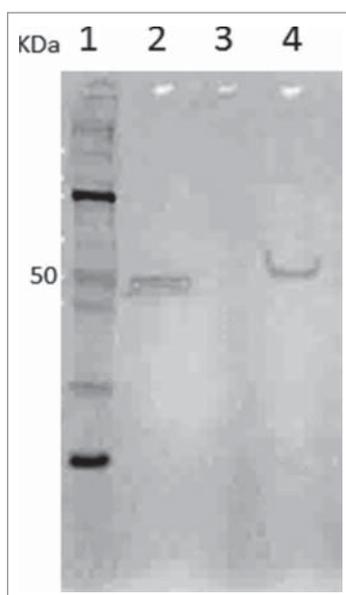
#### Effect of *rTbgTCTP* on bacterial growth

Among the 24 bacteria strains that were shown to bind *rTbgTCTP*, growth kinetics were only obtained

for 14 strains (Fig. 5). Figure 5 shows the bacterial growth in the absence of *rTbgTCTP*, with the respective controls under the different culture conditions (Fig. 5A–C: culture at 25°C under aerobiosis, microaerobiosis, and anaerobiosis, respectively; Fig. 5D–F: similar culture conditions at 37°C). The effect of *rTbgTCTP* was then evaluated with respect to the growth kinetics of this control experiment.

When cultivated at 25°C, most of the tested bacteria strains were not affected by the presence of *rTbgTCTP*, regardless of the concentration (data not shown). Only the strains Gmm09 (*Serratia* sp.), Cam33C (*Enterococcus* sp) and Cam20B (*Providencia* sp) exhibited modified growth kinetics in the presence of *rTbgTCTP* (Fig. 6A).

At 37°C, the effect of *rTbgTCTP* was very diverse depending on the bacterial strain, the culture conditions (aerobiosis, microaerobiosis or anaerobiosis) and the concentration of *rTbgTCTP* (Fig. 6B–E). With the exception of several strains (Cam33A, Cam33C and Cam20B) after 24 h of culture in aerobiosis, the effect of *rTbgTCTP* was stronger when the strains were cultured in anaerobiosis or microanaerobiosis. We observed that the presence of *rTbgTCTP*, no matter the concentration, frequently caused a dramatic decrease in bacterial growth (Fig. 6B–E), with a comparable effect irrespective of the bacterial strain or culture conditions. However, this was not the case for Cam 33C, Cam33A, Cam32A and Cam20B (Fig. 6B), or Gmm11, Ang24C and Cam15B (Fig. 6E). In these cases, growth was stimulated up to 10- or 20-fold the reference of the corresponding control, depending on the *rTbgTCTP* concentration and the culture



**Figure 4.** Binding between bacteria and *rTbgTCTP* as revealed by western blot using anti-His mAb. Lane 1: molecular weight markers (Novex Sharp Pre-Stained Protein Standard, Thermo Fisher Scientific); lane 2: purified *rTbgTCTP*; lane 3: bacteria (*Providencia* spp. from *Glossina palpalis palpalis*) incubated without *rTbgTCTP* as a control; lane 4: bacteria incubated with *rTbgTCTP* and subsequently treated with  $\beta$ -mercaptoethanol.

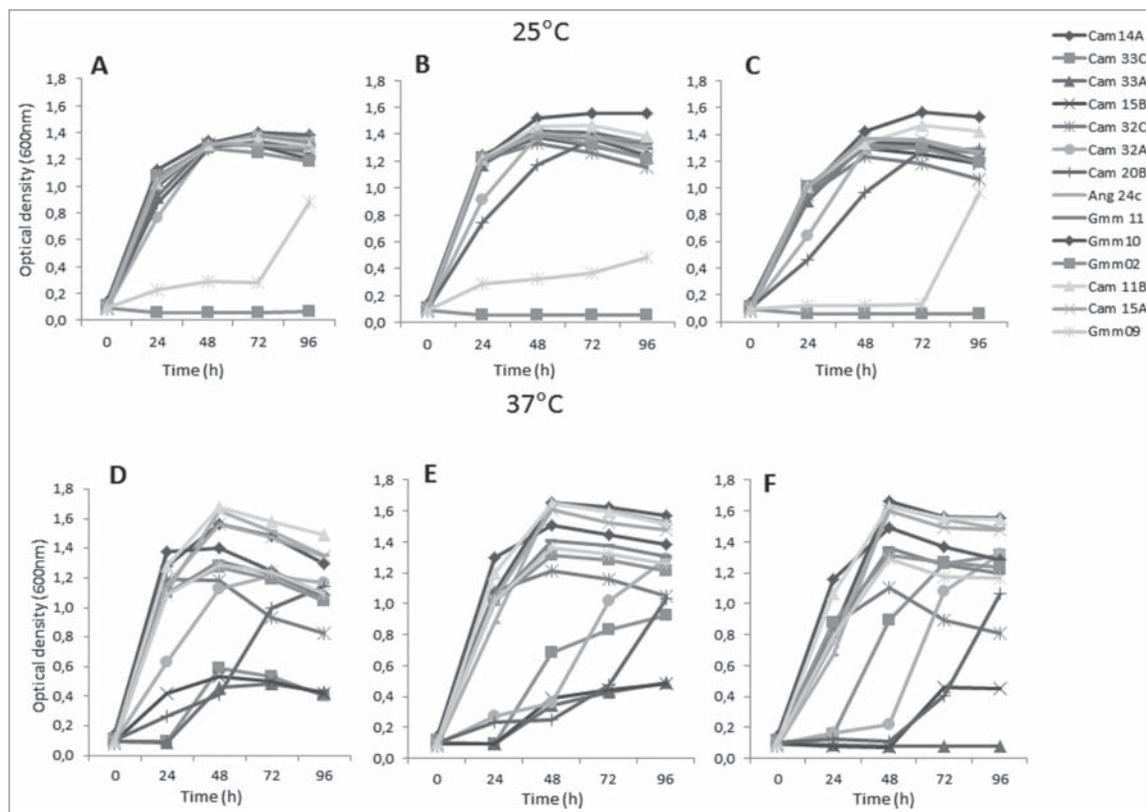
**Table 1.** Designation and origin of the bacteria strains examined in this study. Descriptions include the bacterial identifying code and species, as well as the *Glossina* species from which they were isolated and the country where flies were collected.

Strains	Results Western blot	Bacteria species	Glossina species	Localization
Anne B1	–	Serratia sp.	Glossina palpalis gambiensis	Insectarium
Anne 17B	–	Enterobacter sp.	Glossina palpalis palpalis	Angola_HAT focus of Maria-Teresa (Bengo province)
Cam 29 B	+	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Campo Beach/Ipono
Serratia glossinae	–	Serratia sp.	Glossina palpalis gambiensis	Insectarium
Anne L1	–	Serratia sp.	Glossina palpalis gambiensis	Insectarium
Cam 24 C	–	Enterobacter sp.	Glossina nigrofusca	Cameroon_HAT focus of Campo_Campo Beach/Ipono
Cam 26 C	+	Enterococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_ND
Angola 23 C	+	Serratia sp.	Glossina palpalis palpalis	Angola_HAT focus of Maria-Teresa (Bengo province)
Cam 29 A	+	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Campo each/Ipono
Gmm 11	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Cam 14 A	+	Lactococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Angola 8 B	–	Acinetobacter sp.	Glossina palpalis palpalis	Angola_HAT focus of Maria-Teresa (Bengo province)
Angola 8 A	–	Enterobacter sp.	Glossina palpalis palpalis	Angola_HAT focus of Maria-Teresa (Bengo province)
Cam 33 B	–	Enterococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_ND
Cam 20 A	–	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo
Cam 32 A	+	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo
Cam 24 B	–	Enterobacter sp.	Glossina nigrofusca	Cameroon_HAT focus of Campo
Cam 18 A	–	Enterobacter sp.	Glossina pallicera	Cameroon_HAT focus of Campo
Cam 32 C	+	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo
Cam 32 A	+	Enterococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Campo each/Ipono
Gmm 15	–	Serratia sp.	Glossina morsitans morsitans	Insectarium
Cam 11 B	+	Enterobacter sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Cam 33 C	+	Enterococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Campo each/Ipono
Angola 16 C	+	Enterococcus sp.	Glossina palpalis palpalis	Angola_HAT focus of Maria-Teresa (Bengo province)
Cam 15 A	+	Enterococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Cam 15 B	+	Enterococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Angola 24 C	+	Enterobacter sp.	Glossina nigrofusca	Cameroon_HAT focus of Campo_Campo Beach/Ipono
Gmm 09	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Gmm10	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Gmm 02	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Cam 20 B	+	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Gmm 04	–	Serratia sp.	Glossina morsitans morsitans	Insectarium
Cam 20 C	+	Providencia sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Gmm 07	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Gmm 08	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Gmm 12	–	Serratia sp.	Glossina morsitans morsitans	Insectarium
Cam 14 B	+	Lactococcus sp.	Glossina palpalis palpalis	Cameroon_HAT focus of Campo_Akak
Gmm 05	+	Serratia sp.	Glossina morsitans morsitans	Insectarium
Gmm 06	–	Serratia sp.	Glossina morsitans morsitans	Insectarium

conditions, as shown in [Figure 6C](#) (Cam 32C, Cam 15A and Gmm09) and [Figure 6D](#) (Cam14A, Gmm02, Cam11B and Gmm10). This positive or negative effect of *rTbgTCTP* was detectable with very low concentrations of the protein (*i.e.*, 1.6  $\mu$ M).

## Discussion

TCTP is one of the proteins that we previously demonstrated are excreted/secreted by trypanosomes, whose gene is differentially expressed in



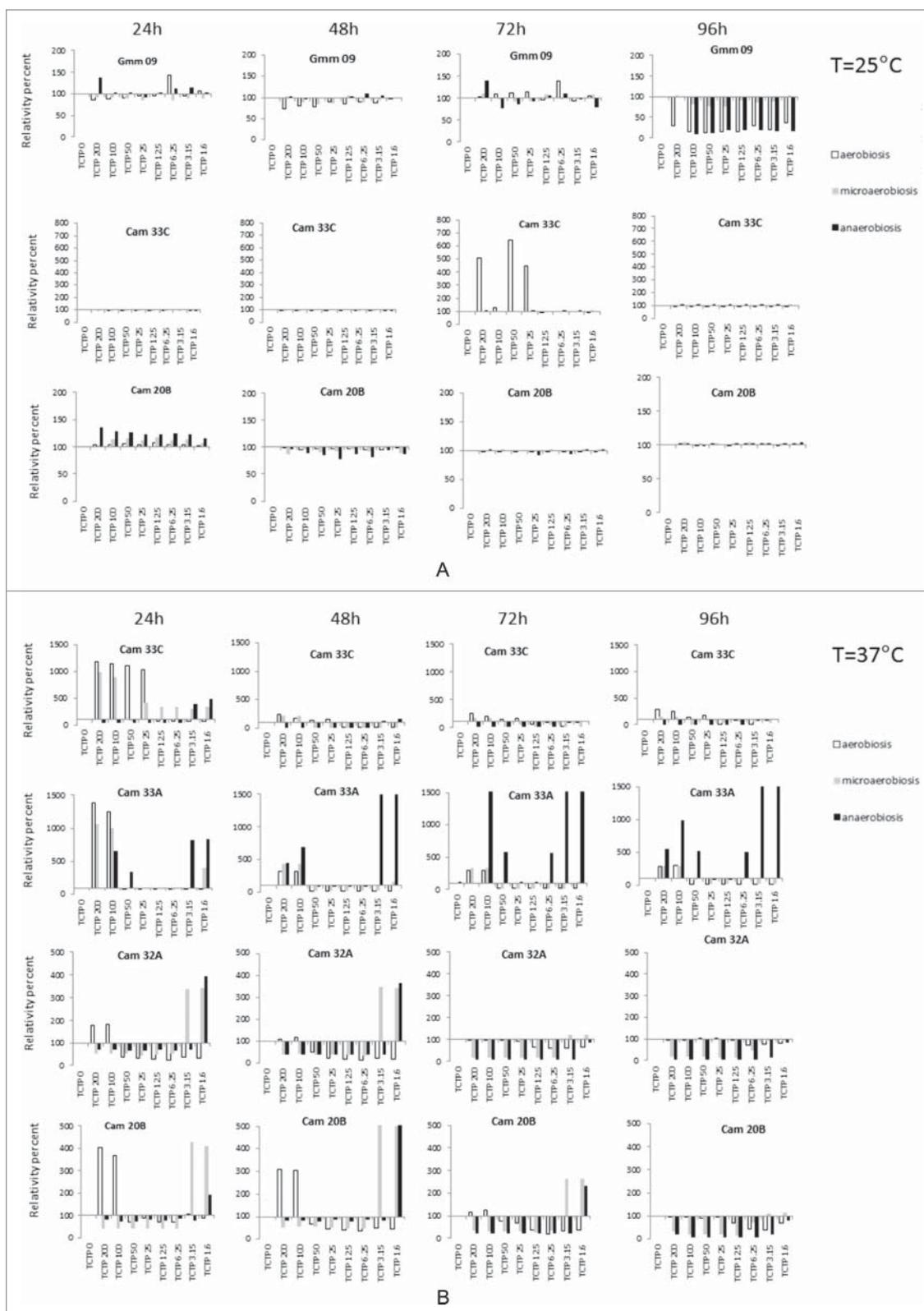
**Figure 5.** Growth kinetics of the bacteria strains grown in the absence of *rTbgTCTP*. The bacterial concentration was measured using the OD at 600 nm. Bacteria were cultivated in Mitsuhashi medium at 25°C (A-C) or 37°C (D-F), in either aerobiosis (A, D), microaerobiosis (B, E) or anaerobiosis (C, F).

trypanosome-infected vs. non-infected *Glossina*. This protein has been reported to play multiple roles, and we investigated its possible effect on bacteria from *Glossina* gut microflora using a recombinant TCTP from *Tbg* along with bacteria isolated from diverse *Glossina* species. This focus on gut microflora was based on the premises that (i) TCTP *in vivo* excretion by trypanosomes should be accompanied by its release in the gut, and that (ii) at least some portion of the midgut bacteria are involved in tsetse vector competence.

First, we fully succeeded in producing a recombinant TCTP that corresponds to the TCTP encoded by *T. brucei gambiense* (GST (glutathione S-transferase) - His-*Tbg* 927.8.6750 protein, referred to here as *rTbgTCTP*). As expected, given the data reported in the literature, the *rTbgTCTP* was very close to its homologs from other trypanosomatids. Of even greater interest was the demonstration that this protein can bind to bacteria isolated from the midgut of different tsetse fly species and interfere with bacteria growth, despite multiple variations in bacteria strain and culture conditions.

### Binding of *rTbgTCTP* with bacteria

Recently, Hansenová Maňásková et al.<sup>32</sup> reported on the incorporation of a protease substrate into the bacterial cell wall. This event is processed by a bacterial cell wall-associated protease and involves the valine-leucine-lysine (VLK) peptidyl sequence of the substrate that acts as an anchor for binding the substrate to the bacterium. Interestingly, the *rTbgTCTP* amino acid sequence comprises a nearly similar peptidyl motif valine-leucine-cysteine-lysine (VLCK), which differs by its inclusion of a cysteine. Further investigations must therefore address whether or not this cysteine could hinder the potential anchor effect attributed to the VLK motif. Nevertheless, the presence of a cysteine moiety in the *rTbgTCTP* primary structure is of great interest, since the -SH function of this amino acid frequently establishes a disulfide covalent bridge (-S-S-) with the -SH of another cysteine. Such a bridge is commonly involved in the formation and maintenance of protein tertiary (requiring the presence of at least 2 cysteines) or quaternary (requiring at least one cysteine in each subunit) conformations. This



**Figure 6.** Effect of different concentrations of *rTbgTCTP* on bacteria growth. The effect was tested at  $25^{\circ}\text{C}$  (A) and  $37^{\circ}\text{C}$  (B-E), under 3 different atmosphere compositions: aerobiosis (white column), microaerobiosis (gray column), and anaerobiosis (black column). Histograms compare, for a given experimental condition (and for each time), the OD of the tested culture (with TCTP at a given concentration) with the OD of the corresponding control culture without TCTP (assigned to the value 100%). The results are the mean of triplicate experiments.

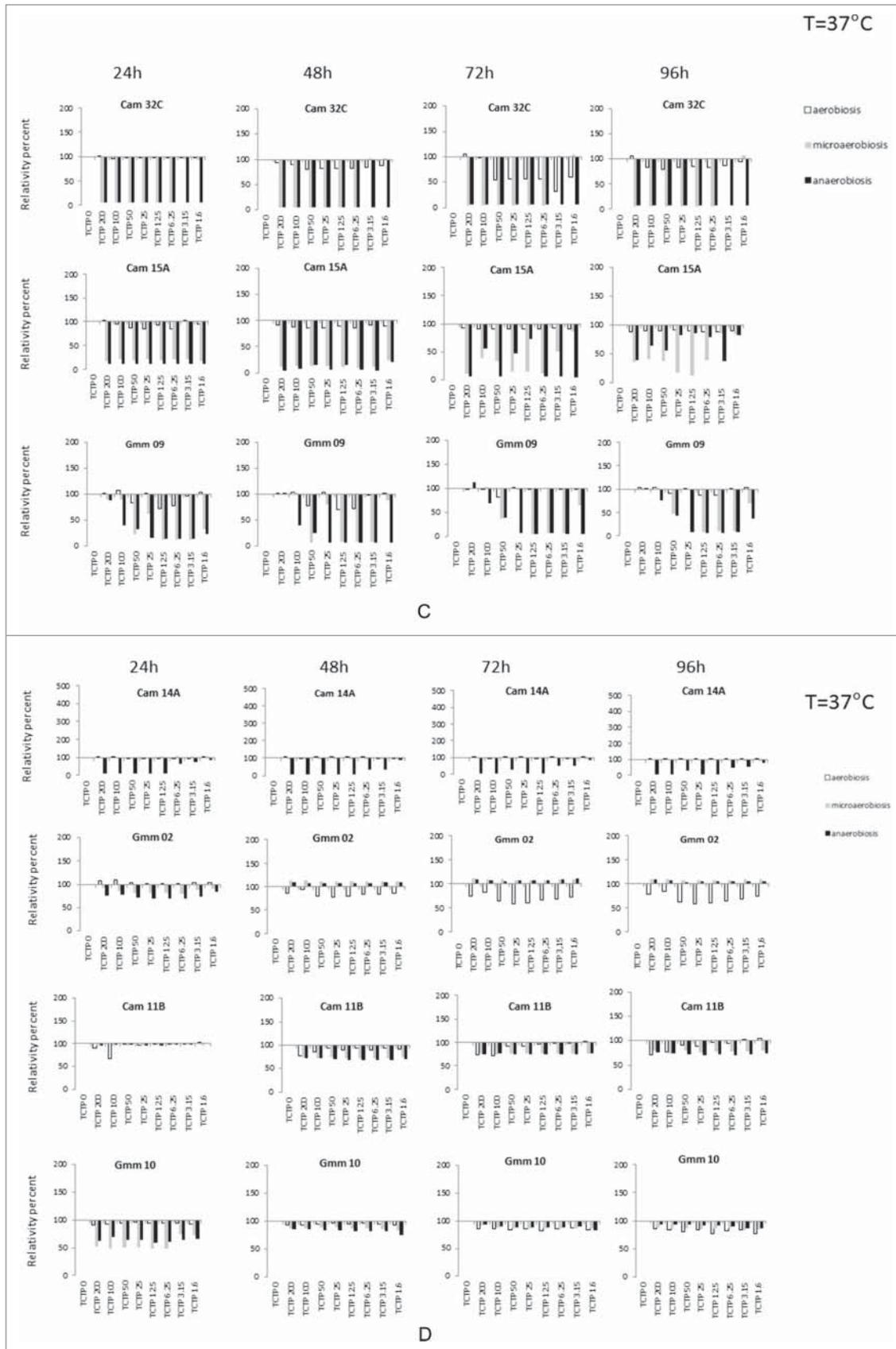


Figure 6. (Continued).

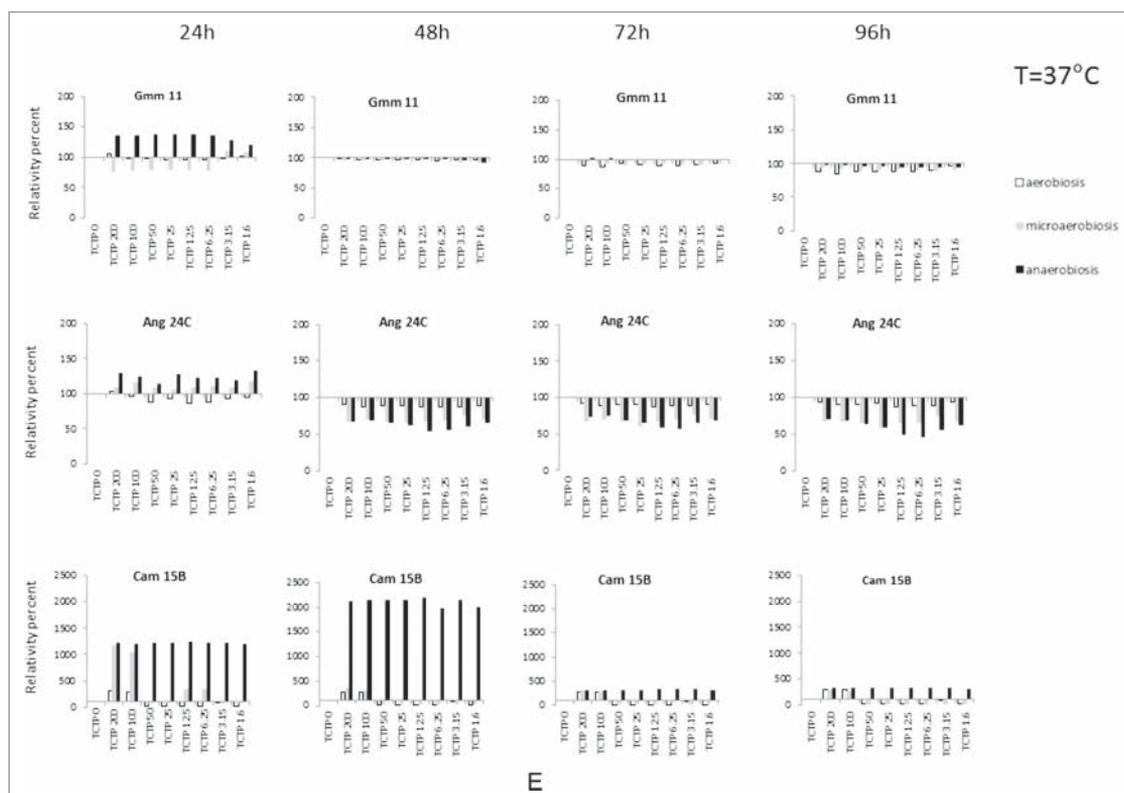


Figure 6. (Continued).

disulfide covalent bond is easily broken by  $\beta$ -mercaptoethanol (or dithiothreitol) treatment. This leads us to believe that the binding of *rTbgTCTP* with bacteria is most probably due to the formation of a disulfide bridge between  $-SH$  groups from cysteines in the *rTbgTCTP* and the bacterial cell wall. This covalent bond is strong enough to maintain the protein/bacterial link during a drastic washing procedure like we used here, although the protein was released when the *rTbgTCTP*/bacterium complex was treated with  $\beta$ -mercaptoethanol, as revealed in our electrophoresis and immunodetection experiments. Another type of binding could be available between the bacterial membrane and *rTbgTCTP*. In this scenario, lysin sites of the *rTbgTCTP* protein, localized on the 3D structural helix with an outside orientation, could play a role in binding to the bacteria membrane, thereby impacting bacteria growth as antimicrobial properties.<sup>33</sup>

### The effect of *rTbgTCTP*

Our results indicate that the effect of *rTbgTCTP* can either inhibit or stimulate the growth of bacteria from the *Glossina* midgut, and that this effect depends

mainly on the bacterium under consideration, the culture conditions and (to a lesser extent) the protein concentration. At this stage of research, it is difficult to determine how certain bacteria strains are sensitive to the presence of TCTP while others, sometimes belonging to the same species, are insensitive to this protein. Similarly, in the case of “susceptible” bacteria, it is difficult to speculate on the factors that direct this effect toward the stimulation or inhibition of bacterial growth. Nevertheless, the presence of *rTbgTCTP* is able to modify the microbial composition of the “ecological niche” constituted by the tsetse fly midgut. Modifying the microbial composition may in turn alter the ability of the fly to transmit the trypanosome responsible for sleeping sickness, given that several intestinal bacteria including *Sodalis glossinidius* were previously shown to be involved in tsetse fly vector competence.<sup>34,35</sup>

TCTP is known to play many physiological roles, raising the question of how its role could seemingly be limited to modifying bacterial growth as reported here. Wang et al.<sup>36</sup> demonstrated the involvement of *Bombyx mori* (*Bm*) TCTP (*BmTCTP*) in the immune response of the silkworm *Bm* to viral infection.

When added to the insect cell culture, *BmTCTP* induced the activation of the extracellular signal regulate kinase (ERK) signaling pathway along with the expression of several genes, including the anti-microbial peptide cecropin. It was previously shown that the tsetse cecropin gene (in addition to many others) is overexpressed in tsetse flies that are refractory to trypanosome infection, e.g. flies that are able to eliminate parasites ingested while taking a blood meal on an infected host.<sup>37</sup> It could be argued that in the *B. mori* example the TCTP acting on the cell culture is the endogenous protein resulting from the expression of the gene encoded by the insect's own genome, whereas in the present study the TCTP that is expected to act on the fly cells is an exogenous protein produced and excreted by the trypanosome. However, this argument may be ruled out to the extent that the TCTP of one organism can supplement the TCTP deficiency of another organism, no matter how genetically distant. Indeed, this ability has been demonstrated by Brioude et al.,<sup>17</sup> who reported on the ability of *Arabidopsis* TCTP to supplement a defect in *Drosophila* TCTP (after disruption of the corresponding gene), and vice versa. Thus, it cannot be excluded that *rTbgTCTP* can "manipulate" genome expression in insect cells, targeted to specific genes including anti-microbial compounds and possibly also genes involved in the fly immune response. This is consistent with the number of proteins that are excreted/secreted by trypanosomes,<sup>38,39</sup> moreover number of genes from the different partners involved in host (tsetse fly), midgut bacteria (e.g., *Sodalis* and *Wigglesworthia*), and the parasite (trypanosome) that have been shown to be differentially expressed.<sup>40</sup> In this context, Aksoy et al.<sup>28</sup> recently reported on the release of the trypanosome coat protein variable surface glycoprotein (VSG) into the tsetse fly gut. The internalization of VSG into the cardia cells causes an interference that ultimately leads to the reduction of the peritrophic matrix efficiency, consequently favoring the crossing of this barrier by the parasite and its establishment in the gut.

In conclusion, trypanosomes have developed a wide range of mechanisms allowing them to establish and survive within the midgut of their insect host, the tsetse fly. *TbgTCTP* may be part of this array, as it could, among other possible effects, eliminate intestinal bacteria, or reduce their growth, some of which being

known to secrete anti-parasitic compounds such as prodigiosin produced by *Serratia* spp and *Enterobacter* spp, toxic to *P. falciparum* and *T. cruzi*,<sup>41,42,43</sup> or active oxygen reported to be produced by *Enterobacter* spp, and to be toxic to *P. falciparum*<sup>44</sup>; this effect would favor trypanosome establishment into the tsetse fly gut. Thus, as such, *TbgTCTP* may constitute a target for controlling the survival and transmission of the trypanosome, with consequences for the mammalian hosts and the proliferation of sleeping sickness.

Future work should continue the investigations on *TbgTCTP* and its potential molecular partners, so as to decipher their role in fly vector competence. Furthermore a question will raise of how to counteract the possible favorable effect of *TbgTCTP* on trypanosome infection and how to manage it in the frame of a disease control strategy in the field. The use of compounds blocking the active site of the protein, or of antibodies anti-*TbgTCTP* could be explored. To deliver such products *in situ*, within the fly gut, a para-transgenic approach such as suggested by Rio et al.<sup>45</sup> could be tested. This would need *Sodalis glossinidius*, a tsetse fly facultative symbiont, to be isolated, cultured *in vitro*, adequately genetically transformed, and transferred (micro-injection) into a female fly harboring *Wolbachia* (W), another secondary tsetse symbiont which confers to the fly a reproductive advantage compared with wild-type (W-) flies. Since both symbionts are maternally transferred to the offspring, the dissemination in a focus of (W+) female tsetse flies hosting the modified *Sodalis* symbiont, will lead to the progressive replacement, over generations, of the natural population by the population of modified tsetse fly. According to Alam et al.,<sup>46</sup> the dissemination of several flies corresponding to 10% of the natural population of a focus should lead to the replacement of 90% of this population just within 2 years, thus decreasing drastically the risk of the sleeping sickness spread. Thus, such approach could open new strategies to improve vector control and the fight against HAT.

## Materials and methods

### *Glossina intestinal* bacteria strains

The bacteria that were tested in our study were previously isolated from different *Glossina* species that were either amplified in the CIRAD's insectarium in Montpellier (France) or collected in HAT foci in

different sub-Saharan African countries. The bacterial strains were preserved in the UMR IRD-CIRAD Inter-trypt cryobank in Montpellier (France) or the IRD-MIO in Marseille (France), and re-cultured as needed to perform the present study. The designation and origin of the bacteria strains are provided in Table 1.

### **Construction, in vitro production and purification of rTbgTCTP**

The *TbgTCTP* gene was synthesized (Genecust Company; Ellange, Luxembourg) according to the *Tbg* 927.8.6750 sequence from the NCBI database. The gene was then cloned into the baculovirus vector pAcGHLT-A using the *EcoRI* and *NotI* restriction sites. Automated DNA sequencing was performed to confirm the integrity of the cloned sequence (GATC Biotech; Constance, Germany).

Two million Sf9 cells were co-transfected with 2  $\mu\text{g}$  of *Tbg*927.8.6750-pAcGHLT-A and 0.5  $\mu\text{g}$  of BD BaculoGold Baculovirus linearized DNA, according to the manufacturer's instructions (BD Biosciences Pharmingen). The presence of the recombinant virus was detected by PCR after viral DNA extraction with QIAmp MiniElute Virus Spin (Qiagen). Infected Sf9 cells were cultured for 48 h, corresponding to the optimum time to reach the highest glutathione S-transferase-His tagged *Tbg* 927.8.6750 (*rTbgTCTP*) expression level (data not shown). The insect cells were collected, lysed with a buffer containing 50 mM Tris HCl, 150 mM NaCl, and nonyl phenoxypolyethoxyethanol (NP40) (1%) at pH 8, incubated on ice for 30 min, and finally sonicated (3 10-s pulses of 12 V, each separated by 6-s intervals). The homogenate was then centrifuged at 14,000 g for 10 min at 4°C, and the supernatant was loaded at a flow rate of 0.5 ml/min onto a 1 ml Ni-NTA agarose column (ÄKTAprime system, GE Healthcare Life Sciences; Little Chalfont, UK) pre-equilibrated with buffer A (20 mM Tris HCl, pH 7.4; 500 mM NaCl; 20 mM imidazole) at room temperature. The column was subsequently washed (flow rate: 1 ml/min) with buffer A until the absorbance at 280 nm decreased to the basal level. The *rTbgTCTP* was then eluted (flow rate: 1 ml/min) with buffer B (20 mM Tris HCl buffer pH 7.4; 500 mM NaCl; 250 mM imidazole). The eluate was first dialyzed with spectra/Por 3 (Spectrumlabs; Rancho Dominguez, California, USA) against phosphate buffer saline (PBS) overnight at 4°C, and then concentrated by dialyzing against polyethylene glycol

3350 Da (Sigma-Aldrich; Darmstadt, Germany). The protein content of the concentrated eluate was quantified using the Bradford method (Pierce Coomassie Protein Assay Kit, Thermo Fisher; Waltham, Massachusetts, USA), and the purity of the eluted protein was verified after electrophoresis on 10% polyacrylamide SDS-PAGE gels<sup>47</sup> stained with PAGE blue staining solution (Thermo Scientific; Waltham, Massachusetts, USA).

### **Western blot analysis**

SDS-PAGE gels were blotted onto a 0.45- $\mu\text{m}$  nitrocellulose<sup>TM</sup> membrane (Bio-Rad; Hercules, California, USA). Membranes were then incubated for 1 h at room temperature in 5% (w/v) nonfat skim milk agitated with shaking at 450 rpm. Next, the membranes were washed 3 times for 5 min with PBS containing 0.05% Tween 20. Finally, the membranes were incubated for 1 h at room temperature in PBS containing 5% nonfat skim milk and a 1:2000 diluted mouse anti-His tag antibody conjugated to horseradish peroxidase (Sigma-Aldrich; Darmstadt, Germany). After 3 washes of 5 min in PBS containing 0.05% Tween 20, membranes were developed using the Luminata crescendo system (Millipore; Molsheim, France).

### **Bioinformatics analysis**

Several bioinformatics tools were used to investigate the structural characteristics and biochemical properties of the *rTbgTCTP*. 3D structure analysis was performed with the Chimera software. The CBS prediction servers were used to predict the post-translational modifications of *rTbgTCTP*, especially the N-linked glycosylation (NetNGlyc 1.0 Server)<sup>48</sup> and O-glycosylation (NetOGlyc 4.0 Server)<sup>49</sup> sites, but also immunological features such as Linear B-cell epitopes (Bepipred 1.0 Server)<sup>50</sup> and discontinuous B-cell epitopes (DiscoTope 2.0 Server).<sup>51</sup> The PhyML website was used to construct a phylogenetic tree based on differences in the amino acid sequences between TCTP and several trypanosomatids (including *Tbg*) as well as several mammalian and *Glossina* species.<sup>52-55</sup>

### **Interaction between TCTP and bacteria from *Glossina* midguts**

In collaboration with the IRD-MIO team (Marseille, France), a total of 39 bacteria strains were obtained

for use in this study. This includes species that we previously identified such as *Acinetobacter* sp, *Serratia* sp, *Enterobacter* sp, *Enterococcus* sp, *Lactococcus* sp, and *Providencia* sp, as well as species isolated from different *Glossina* species sampled in different countries such as *G. p. palpalis* (Angola and Cameroun), *G. m. morsitans* (CIRAD insectarium; Montpellier, France), *G. pallicera* (Cameroun), *G. nigrofusca* (Cameroun), and *G. palpalis gambiensis* (CIRAD insectarium; Montpellier, France).<sup>14,29,31</sup> To test the interaction between rTbgTCTP and the bacteria, 5 µg of the protein were added to 100 µl of freshly cultured bacteria displaying an optical density (OD) of 0.3 at 600 nm, to obtain a 0.1 OD to start the growth kinetic for each bacterium tested; samples were then incubated 2 h at 25°C. The incubation medium was then centrifuged at 12,000 g for 10 min, after which the supernatant was discarded and the pellet (comprising bacteria coupled or not to rTbgTCTP) was washed 4 times in 500 µl PBS. The final pellet was re-suspended in 10 µl PBS. The bacteria-only control was processed similarly. β-mercaptoethanol (1 µl) and 10 µl buffer were added to each sample to reduce covalent disulfide bonds, and finally 10 µl of 2X loading buffer containing sodium dodecyl sulfate (SDS), glycerol and running buffer (Trizma base, SDS, Glycin) were added to the samples that had been boiled for 3 min to denature the rTbgTCTP. Samples (21 µl) were loaded onto a 10% SDS acrylamide gel, and after electrophoresis (30 mA for 1 h) the protein bands were transferred onto a 0.45 µm nitrocellulose<sup>TM</sup> membrane (Bio-Rad) and processed as described in section 1.3, to detect the presence of rTbgTCTP.

### **Effect of rTbgTCTP on the growth of bacteria isolated from *Glossina* midguts**

To evaluate the effect of rTbgTCTP on bacteria growth, a solution containing 150 µl of 'Mitsubishi and Maramorosch' insect culture medium, 50 µl of each bacterium pre-culture and 10 µl of rTbgTCTP at different concentrations (sterilized by filtration on a 0.22 µm membrane) were mixed in one well of a 96-well NUNC Elisa plate. The final rTbgTCTP concentration in the cultures ranged from 1.6 – 200 µM (0 µM for the control culture). This protocol was performed in triplicate at 25°C and 37°C, under 3 controlled atmosphere conditions: aerobiosis, microaerobiosis (atmosphere enriched with 5% CO<sub>2</sub>) and anaerobiosis. These

different growth conditions have been chosen a) since in HAT foci the temperature may vary from around 22°C up to over 35°C thus influencing the corporeal temperature of the tsetse fly which is a poikilotherm invertebrate (its corporeal temperature is not fixed and stable but depends on the temperature of its environment), and b) since the concentration in oxygen in the fly intestine (from which the different bacteria have been isolated) may vary from near anaerobic to micro-aerophilic conditions; the aerobiosis atmosphere has been tested to complete the spectrum. The evolution of bacterial growth was estimated by measuring the culture optical density (OD) at 600 nm at 0, 24, 48, 72 and 96 h after incubation, using an Enspire spectrophotometer (Perkin Elmer; Waltham, Massachusetts, USA). At t = 0, each bacterial culture was adjusted to OD = 0.1 at 600 nm. To present the effect of TCTP, histograms were constructed by comparing, for a given experimental condition (and for each time), the OD of the tested culture (with TCTP at a given concentration) with the OD of the corresponding control culture (without TCTP; arbitrarily assigned to 100%). This procedure facilitates comparisons across all culture conditions and times of growth.

### **Disclosure of potential conflicts of interest**

No conflicts of interest were disclosed.

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