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SPECIAL ISSUE ARTICLE

Evaluation of two candidate molecules—TCTP and cecropin—on the establishment of *Trypanosoma brucei gambiense* into the gut of *Glossina palpalis gambiensis*

François Sougal Ngambia Freitas[1,2], Linda De Vooght[3], Flobert Njiokou[1], Jan Van Den Abeele[3], Géraldine Bossard[2], Bernadette Tchicaya[2], Rosa Milagros Corrales[4], Sophie Ravel[2], Anne Geiger[2] and David Berthier-Teyssedre[2]

[1]*Laboratory of Parasitology and Ecology, Department of Animal Biology and Physiology, Faculty of Science, University of Yaoundé I, Yaoundé, Cameroon;* [2]*IRD-CIRAD, UMR INTERTRYP, Univ Montpellier, Cirad, IRD, Montpellier, France;* [3]*Department of Biomedical Sciences, Unit of Veterinary Protozoology, Institute of Tropical Medicine Antwerp, Antwerp, Belgium and* [4]*MIVEGEC, University of Montpellier, Montpellier, France*

Abstract Trypanosomiasis, transmitted by tsetse flies (*Glossina* spp.), poses a significant health threat in 36 sub-Saharan African countries. Current control methods targeting tsetse flies, while effective, allow reinfestation. This study investigates paratransgenesis, a novel strategy to engineer symbiotic bacteria in tsetse flies, *Sodalis glossinidius*, to deliver anti-trypanosome compounds. Disrupting the trypanosome life cycle within the fly and reducing parasite transmission could offer a sustainable solution for trypanosomiasis control. In this context, we tested the effect of cecropin, reported to be lethal for *Trypanosoma cruzi* (Chagas disease) and *Tbg*TCTP (Translationally Controlled Tumor Protein from *Trypanosoma brucei gambiense*), previously reported to modulate the growth of bacteria isolated from the fly microbiome, to delay the first peak of parasitemia and the death of trypanosome-infected mice. We have successfully cloned and transfected the genes encoding the two proteins into *Sodalis* strains. These *Sodalis* recombinant strains (rec*SodalisTbgTCTP* and rec*Sodaliscecropin*) have been then microinjected into the L3 larval stage of *Glossina palpalis gambiensis* flies. The stability of the cloned genes was checked up to the 20th day after microinjection of rec*Sodalis*. The rate of fly emergence from untreated pupae was 95%; it was reduced by nearly 50% due to the mechanical injury caused by microinjection. It decreased to nearly 7% when larvae were injected with rec*SodalisTbgTCTP*, which suggests TCTP could have a lethal impact to larvae development. When challenged with *T. brucei gambiense*, a slightly lower, but statistically non-significant, infection rate was recorded in flies harboring rec*Sodaliscecropin* compared to control flies. The effect of rec*SodalisTbgTCTP* could not be measured due to the very low rate of fly emergence after corresponding treatment of the larvae. The results do not allow to conclude on the effect of cecropin or TCTP, delivered by para-transgenesis into the fly's gut, on the fly infection by the trypanosome. Nevertheless, the results are encouraging insofar as the technical approach works on the couple *G. p. gambiensis*/*T. brucei gambiense*. The next step will be to optimize the system and test other targets chosen among the ESPs

Correspondence: Anne Geiger, IRD-CIRAD, UMR INTERTRYP, Univ Montpellier, Cirad, IRD Campus International de Baillarguet, TA A17/G, 34398 Montpellier Cedex 05, France. Email: anne.geiger@ird.fr

(Excreted-Secreted Proteins) of the trypanosome secretum, or the differentially expressed genes associated with the sensitivity/resistance of the fly to trypanosome infection.

Key words paratransgenesis; *Sodalis glossinidius*; *Trypanosoma*; tsetse flies

Introduction

Vector-borne diseases that severely affect human health represent more than 17% of all infectious diseases, causing more than 700 000 deaths a year according to the World Health Organization (WHO, 2023a). Among them, Human African Trypanosomiasis (HAT/sleeping sickness) causes a heavy burden for the populations of the 36 sub-Saharan countries where it occurs. HAT is caused by flagellated protozoa belonging to the *Trypanosoma* genus transmitted to humans by the tsetse fly (*Glossina* sp) in which the parasite multiplies and achieves part of its biological life cycle. Alongside this, Nagana, the Animal form of trypanosomiasis (Animal African Trypanosomosis/AAT) is widespread over the same countries, causing damage mainly to livestock, and other domestic animals, with strong repercussions on human nutrition; economic losses to African agriculture are estimated at more than 4 billion US$ (Welburn & Maudlin, 2012; Shaw *et al.*, 2013).

In the absence of vaccines, the lack of effective drugs and the development of resistance to trypanocide drugs (Delespaux *et al.*, 2008), the most efficient disease prevention alternative strategies are based on vector control: use of Tiny Targets (insecticide-impregnated cloth pieces) or traps and/or dissemination of sterilized male tsetse flies (SIT-Sterile Insect Technique) in order to reduce and possibly eradicate tsetse flies (Vreysen, 2001).

The limitations of AAT for *T. brucei* control are evident despite treatment programs in domestic animals. A recent survey in Northern Cameroon highlights this challenge. The study found high prevalence both in tsetse flies (45%) and cattle (40%) (Oumarou *et al.*, 2022; Farikou *et al.*, 2023). These findings demonstrate the continued circulation of the parasite even with AAT control implementation.

Regarding HAT, at the turn of the century, about 30 000 new cases per year were reported which led WHO (2002), the Pan-African Tsetse and Trypanosomiasis Eradication Campaign (PATTEC, 2018) and the London Declaration on Neglected Tropical Diseases (2012) (Simarro *et al.*, 2008; Simo *et al.*, 2014) to target the disease for elimination as a public health problem by 2020 and a goal of interrupting transmission by 2030. The initiative to reinforce HAT surveillance and control in all endemic countries has led to a progressive reduction in incidence in the ensuing years, reaching less than 1000 annual cases since 2018 (WHO, 2023b). Despite this success, the estimated population at risk for the period 2016–2020 was 55 million people, of whom only 3 million were at moderate-high risk (WHO, 2023b). Thus, if favorable conditions, or impaired control activities, occurred, an increase in the severity of the HAT epidemic is to be feared (Mehlitz & Molyneux, 2019). Such an increase was recorded in Guinea after the Ebola epidemic (2014–2015) during which trypanosomiasis monitoring had to be interrupted (Camara *et al.*, 2017; Camara *et al.*, 2021).

Several hypotheses have been suggested, one of which is the existence of domestic and wild animal reservoirs of trypanosome species including *T. brucei gambiense*, as reported in numerous studies (Njiokou *et al.*, 2004; Simo *et al.*, 2006; Simo *et al.*, 2008; Farikou *et al.*, 2010; N'Djetchi *et al.*, 2017; Grébaut *et al.*, 2020). Another hypothesis is the existence of a human reservoir that escapes the screening/treatment strategies (Capewell *et al.*, 2019). Finally, the possibility of reinfestation of controlled foci by infected flies from uncontrolled foci scattered throughout the forest has also to be considered (Farikou *et al.*, 2011).

Faced with these potential risks, complementary or alternative strategies need to be developed. One of these could consists in the suppression of the tsetse fly's vector competence by making it refractory to trypanosome infection or by interrupting the life cycle that the parasite needs to complete within its vector. As the tsetse fly is viviparous, it is not possible to consider the genetic transformation of the tsetse fly itself, which would require using its germ cells. Thus, an alternative approach, paratrangenesis, suggested by Rio *et al.* (2004), is therefore being tested. After suitable engineering, it uses *Sodalis glossinidius*, the *Glossina* fly's secondary symbiont, to deliver anti-trypanosomal compounds into the fly. As summarized by Geiger *et al.* (2018) several features make *S. glossinidius* a good tool for achieving paratransgenesis while preserving the environment after dissemination of the flies harboring the recombinant symbiont (rec*Sodalis*). Briefly, *S. glossinidius* can be isolated from the tsetse fly, *in vitro* cultivated, genetically engineered, and reintroduced into the fly; it is maternally transmitted to offspring. In addition, *S. glossinidius* is metabolically dependent on the tsetse fly which suggests that no gene flow will occur toward any other organisms.

All of these features, including the fact that tsetse flies benefit of a reproductive advantage when mating includes *Wolbachia*-positive females, should allow to achieve the goal of the paratransgenic approach, i.e. to produce a trypanosome-refractory tsetse (W+) phenotype capable, after dissemination into THA or TAA foci, of progressively replacing susceptible natural populations (Alam *et al.*, 2011). This would generate "self-sustaining" vector control system with no risk of dissemination of the transgene nor modifying the biological diversity of the ecosystem, with the exception of trypanosomes. Furthermore, this control strategy will fit with the WHO's "One Health" recommendation.

Paratransgenesis has been successfully used to express various *S. glossinidius* transgenes into the *Glossina morsitans morsitans* fly, the vector of *T. brucei rhodesiense* causing the acute form of THA in sub-Saharan East African countries (De Vooght *et al.*, 2014; De Vooght *et al.*, 2018; De Vooght *et al.*, 2022; Yang *et al.*, 2022). In contrast, to date no similar experiment has yet been carried out on the *S. glossinidius–G. palpalis gambiensis* couple, the fly species, which transmits *T. b. gambiense* responsible for the chronic form of the disease in Western Africa. The present study aims to fill this gap. It includes three main steps: (i) the genetic transformation of *S. glossinidius* with the *Translationally Controlled Tumor Protein* (*TCTP*) and *cecropin* genes independently and the control of the expression of the transgenes; (ii) the microinjection of the two recombinant *Sodalis* strains (rec*Sodalis*) into *G. p. gambiensis* at their L3 larval stage and its impact on the fly emergence; and (iii) the possible effect of rec*Sodalis* on the susceptibility/refractoriness of the flies to *T. b. gambiense* infection.

The ubiquitous protein TCTP has been implicated in a wide range of cellular processes across various organisms. Studies have documented its involvement in stress responses (Brioudes *et al.*, 2010), cell proliferation (Stierum *et al.*, 2003), even playing a role in embryo development (Roque *et al.*, 2016). Conversely, TCTP has also been linked to apoptosis or programmed cell death (Susini *et al.*, 2008). Interestingly, recent research suggests a potential role for TCTP in physiological disorder (Kadioglu & Efferth, 2016; Seo & Efferth, 2016), highlighting the complex and multifaceted nature of this protein's function (Chen *et al.*, 2013).

As such, TCTP has been considered as a potential therapeutic target against several physiological disorders or diseases (Eichhorn *et al.*, 2013; Kadioglu *et al.*, 2016; Seo & Efferth, 2016). Moreover (i) it is part of the trypanosome secretome (Atyame Nten *et al.*, 2010); (ii) it can be detected in the blood of trypanosomes-infected rats (Geiger *et al.*, 2010); and (iii) the corresponding gene is expressed in infected tsetse flies (Geiger *et al.*, 2015; Hamidou Soumana *et al.*, 2015). Finally, it has been shown that recombinant TCTP modulates the *in vitro* growth of bacteria isolated from the intestine of the fly (Bossard *et al.*, 2017) and that mice immunization with TCTP makes it possible to delay the first peak of parasitemia and the death of mice by a few days (Bossard *et al.*, 2021).

Cecropin has been reported to be lethal for *T. cruzi*, responsible for Chagas' disease. This parasite is transmitted to humans by the hematophagous insect *Rhodnius prolixus* which harbors a symbiont, *Rhodococcus rhodnii*. When this symbiont, after transformation to express *cecropin*, was ingested by *R. prolixus*, the latter became unable to transmit the parasite (Durvasula *et al.*, 1997; Beard *et al.*, 2001; Beard *et al.*, 2002). Furthermore, Kim *et al.* (2004) showed that manipulation of the endogenous immune response of *Anopheles gambiae*, such as the temporal and spatial expression of *cecropin*, may result in a refractory phenotype; more specifically, the midgut-specific activation of Cecropin A in the transgenic *A. gambiae* resulted in 60% reduction of *Plasmodium* oocysts. Similarly, Kokoza *et al.* (2010) showed that transgenic mosquitoes over-expressing both Cecropin A and Defensin A rendered the mosquitoes resistant to *Plasmodium gallinaceum*.

We therefore decided to use the paratransgenic approach to test the effect of *cecropin* and *TCTP* on the transmission of *T. b. gambiense* by *G. p. gambiensis*.

One may note that further vector control using paratransgenesis in wild fly population will be done in combination with SIT method.

Materials and methods

Glossina species

The present study was carried out on *G. p. gambiensis* flies maintained and multiplied in the insectarium of the Centre de Coopération Internationale en Recherche Agronomique pour le Développement (CIRAD) at Montpellier (France), which is part of the Vectopole Sud (https://doi.org/10.18167/infrastructure/00001). The tsetse flies were maintained at 24 °C and 75% relative humidity and fed three times a week (Monday, Wednesday and Friday) with defibrinated sheep blood using an artificial membrane system developed at the Joint research unit INTERTRYP (Montpellier, France).

Bacterial strains and culture conditions

Strains of *Sodalis glossinidius*, the tsetse fly secondary symbiont used in this study were isolated from the haemolymph of surface sterilized *Glossina morsitans morsitans* flies from the colony maintained at the Institute of Tropical Medicine (Antwerp, Belgium). *Sodalis glossinidius in vitro* cultures were carried out at 26 °C in an insect culture medium composed of the Mitsuhashi-Maramorosch Liquid Insect Medium (MM) (PromoCell®) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). Blood agar plates were supplemented with 10% packed horse blood cells and yeastolate (5 mg/mL). All solid cultures were performed at 26 °C under micro-aerophilic conditions generated by the Campygen pack system (Oxoid) which provided 5% O_2, 10% CO_2, balanced with N_2. Antibiotics, either ampicillin (100 μg/mL) or kanamycin (50 μg/mL), were added when selecting recombinant bacteria carrying either the pGRP25 or the pCM66 plasmid, respectively.

Construction of plasmids containing either the TbgTCTP or the cecropin gene

In this study a 6 × His-tag sequence was fused to the *cecropin* (*Cec*) gene by polymerization chain reaction (PCR) and cloned into the *NotI* and *XhoI* sites of the pGRG25 plasmid; this construct is referred to as pGRG25*Cec*. The plasmid pGRG25 (McKenzie & Craig, 2006) bears the *tnsABCD* genes expressed under the control of the *pBAD* promoter and carry a multiple cloning site, flanked by the left and right ends of *Tn7*. The *Tn7* transposition machinery allows for the site-specific transposition at the *attTn7* locus of *Sodalis*; the *tnsABCD* are outside the transposon ends, so that they will not be inserted in the chromosome during the transposition. The plasmid carries araC to mediate arabinose-inducible expression of *tnsABCD* genes. The *TbgTCTP* gene was amplified as a *XbaI-EcoRI* fragment (primers were constructed by integrating at both ends the sequences of *XbaI* and *EcoRI* as respective restriction sites for 5' and 3' parts) by PCR and was cloned into the multiple cloning sites of the pCM66 expression vector (Marx & Lidstrom, 2001) between the *XbaI-EcoRI* sites after enzymes digestion of the PCR product and the plasmid; this construct is referred to as pCM66r*TbgTCTP*. The modified pCM66 harboring the *lac* promoter were then cultured in the presence of 0.1% of Isopropyl β-D-1-thiogalactopyranosid (IPTG) to allow a strong expression of the transgene in a constitutive manner due to the absence of a *Sodalis* lac repressor (De Vooght *et al.*, 2018). All plasmids were sequenced to confirm the presence of the expected DNA sequence and the correct reading frame (we used the T7 fw/rev primers for the pGRG25Cec and the M13 fw/rev for the pCM66r*TbgTCTP*); the primers used for the PCR amplification are shown in the Table 1.

Genetic transformation of Sodalis glossinidius

In order to multiply the plasmid constructs, they were transferred into *Escherichia coli* (TOP10 and DH5α) and the bacteria were cultured until the beginning of the stationary phase (OD_{600} 1.5–2). Plasmid constructs were then extracted and transferred into wildtype (WT) *S. glossinidius* cells using a heat-shock method (Beard *et al.*, 1993) as described by De Vooght *et al.* (2012). Transformed cells were allowed to recover overnight at 26 °C in liquid MM medium prior plating onto MM-blood agar, supplemented with kanamycin (for *S. glossinidius* carrying the pCM66r*TbgTCTP* plasmid) or ampicillin (for *S. glossinidius* carrying the pGRG25*Cec* plasmid). Then, a single recombinant *S. glossinidius* colony was harvested and inoculated into liquid medium and cultured until the beginning of the stationary phase (OD_{600} 0.5–0.6). Prior to the introduction of the rec*Sodalis* into tsetse flies, the expression profile of vector plasmids and the plasmid stability in both *E. coli* and rec*Sodalis* were verified. Secretion of the target proteins was monitored by Western blot analysis from cultures grown up to the beginning of stationary phase.

Western blot analysis

The recombinant bacteria cultures were centrifuged at 3000 r/min for 15 min. The supernatants were then removed and the pellets were re-suspended in 500 μL of a lysis buffer (10 mmol/L HCl + 100 mmol/L NaCl + 3 mmol/L imidazole for a final volume of 50 mL). Subsequently, the samples were then incubated on ice for 30 min after which the samples were lysed by sonication still on ice. Finally, the lysates were centrifuged at 13 000 r/min for 10 min and the supernatants were recovered. Before the migration of the samples, they were concentrated and 15 μL of each sample were mixed with 5 μL of 2 × Load buffer (with β-mercaptoethanol) and 1 μL of DDT (at 100 μg/mL) and the protein were denaturated by heating at 95 °C for 3 min. After proteins denaturation, 20 μL of each sample were loaded on SDS-PAGE gel [10% for *Tbg*TCTP (50 kDa) separation and 20% for cecropin (∼5 kDa)]. The electrophoretic separation was run under 200 V during 2 h. The protein bands were

Table 1 Primer sequences used in the study.

Primer names	Primer sequences (5′–3′)
r*TbgTCTP_Fwpcm66*	TCTAGAATGAAATACCTATTGCCTACGGCAGCCGCT-GGATTGTTATTACTCG
	CGGCCCAGCCGGCCATGGCTAAGATCTTCAGGGAT-ATCTTAACAAATGCC
r*TbgTCTP_pcm66rev*	GAATTCTCACACGCGTTCGCCCTTGAGACCAT
*Cec*_Fw	AATTTTTATAAAATTTTCGTATTCA
*Cec*_Rev	TTATCCTCTAACAGTAGCGGCA
*Tn7*_Fw	TAATACGACTCACTATAGGG
T7-Rev	GCTAGTTATTGCTCAGCGG
*M13*_Fw	GTAAAACGACGGCCAG
*M13*_Rev	CAGGAAACAGCTATGAC
TBR1	CGAATGAATATTAAACAATGCGCAG
TBR2	AGAACCATTTATTAGCTTTGTTGC
*rTbgTCTP*_Fw	ATGAAGATCTTCAGGGATATCTTA
*rTbgTCTP*_Rev	TCACACGCGTTCGCCCTTGAGA
*RecSodCWSP*_Fw	AATTTTTATAAAATTTTCGTATTCA
*RecSodCWSP*_Rev	TTATCCTCTAACAGTAGCGGCA

then transferred on a PVDF membrane at 4 °C for 15 min at 100 mA. After blotting, the PVDF membrane was incubated for 1 h in a 1 × PBS tween solution containing 5% skim milk. After being blocked, the western blot membrane was subsequently incubated overnight with a primary antibody solution (5 mL of 1 × PBS tween solution + 5% skim milk + antibody). For the *Tbg*TCTP revelation, we added 50 μL of anti-mouse TCTP (i.e., 1/100 final dilution); for the cecropin revelation we added 5 μL of anti-His-HRP conjugate (i.e., 1/1000 final dilution). Regarding the cecropin detection we adopted the chemiluminescence approach which was shown (preliminary assays) to be much more sensitive (femtogram range) than the colorimetric one (pictogram range).

Microinjection of recSodalis at the tsetse larval stage

Third-instar larvae of tsetse flies (viviparous insects) were directly injected with 10^6 CFUs (colony forming units) of recombinant *Sodalis* immediately following larviposition. A microinjection technique utilizing a 5 μL Hamilton 75RN syringe with gauge 34 removable electrotapered needles (De Vooght *et al.*, 2018) delivered the bacteria. The injected pupae were maintained in an incubator under controlled temperature (24 ± 1 °C) and humidity (85% relative humidity) for adult emergence (∼30 d). Larval development was monitored by measuring weight before and 2 d after injection to assess the impact of microinjection. Three control groups were employed: a non-injected control (C_ØMI) representing wild-type flies, and two microinjected controls receiving sterile physiological water (C_Cec_MI-H_2O and C_TCTP_MI-H_2O). These "MI-H_2O" controls were necessary to account for potential effects of the microinjection procedure itself, as experiments involving *cecropin* and *TCTP* were performed at separate times.

Infective feeding of tsetse flies from experimental and control batches

Upon adult emergence from pupae, tsetse flies received their first infective meal following a standardized protocol. Cages containing up to 40 flies each were offered a 15-min blood meal from the abdomens of anesthetized mice previously infected with *Trypanosoma brucei gambiense* (ABBA strain). The average parasitemia in these mice was AL 8.1 ± 0.7.

Following the infective meal, flies were fed with non-infective blood meals three times a week for 15–30 min per session on an artificial membrane system. This later was filled with healthy sheep blood pre-warmed at 37 °C. Feeding lasted until day 20, allowing ingested trypanosomes from the initial infective meal to establish within the fly's gut.

Analysis of the effect of recombinant proteins on the parasite establishment in tsetse flies

On day 20 post-infective feed (D20), all flies were dissected to examine gut contents for motile (alive) trypanosomes using phase-contrast microscopy at 400× magnification. To confirm parasite presence with higher

sensitivity, guts were extracted using the Qiagen Dneasy Kit (following manufacturer's instructions) and subjected to PCR analysis. The TBR1/TBR2 primer set was used with a cycling program of: 94 °C for 3 min (initial denaturation), 44 cycles [94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 60 s (extension)], followed by a final extension at 72 °C for 10 min. Negative controls (no template) were included in each PCR run to prevent misinterpretations due to contamination.

We also verified the presence of recombinant symbionts via PCR amplification of the sequences of genes of interest using primer sets (*rTbgTCTP*_Fw/*rTbgTCTP*_Rev and *RecSodCWSP*_Fw/*RecSodCWSP*_Rev) (Table 1) amplifying respectively the sequences of genes encoding *TbgTCTP* and *cecropin* with the following program: (i) initial denaturation at 95 °C for 3 min; (ii) 40 cycles, including 1 cycle consisting in the denaturation at 95 °C for 30 s, annealing at 48 °C (for the *TbgTCTP*) or 43 °C (for the *cecropin*) for 30 s, and extension at 72 °C for 60 s; (iii) final extension at 72 °C for 10 min. Each PCR run included a negative (no-template) control.

Statistics

The Student's *t*-test, Kruskal–Wallis and Chi^2 tests were used for the comparison of the different groups with significant *P*-value ≤ 0.05.

Results

In vitro culture of recombinant Sodalis and verification of the production of recombinant proteins

Recombinant *Sodalis glossinidius* strains harboring pCM66r*TbgTCTP* and pGRG25*Cec* plasmid (Fig. 1) reached stationary phase after approximately 24 h. These cultures where then processed as described in the methodology section to confirm successful transformation and transgene expression (*TbgTCTP* and *cecropin*).

Sanger sequencing confirmed the integration of the pCM66r*TbgTCTP* plasmid and pGRG25*Cec* plasmid into the recombinant *Sodalis* (data not shown), encoding the respective proteins (*Tbg*TCTP and cecropin). Western blot analysis (Fig. 2B) further confirmed the presence of both recombinant proteins. Interestingly, TCTP was detected in both bacterial culture medium and cells extracts, suggesting potential secretion. Induction of *E. coli* pCM66r*TbgTCTP* plasmid with IPTG produced a distinct band at 100 kDa, confirming TCTP expression, compared to negative controls (without induction). However, and surprisingly, the size—100 kDa—of the detected pro-

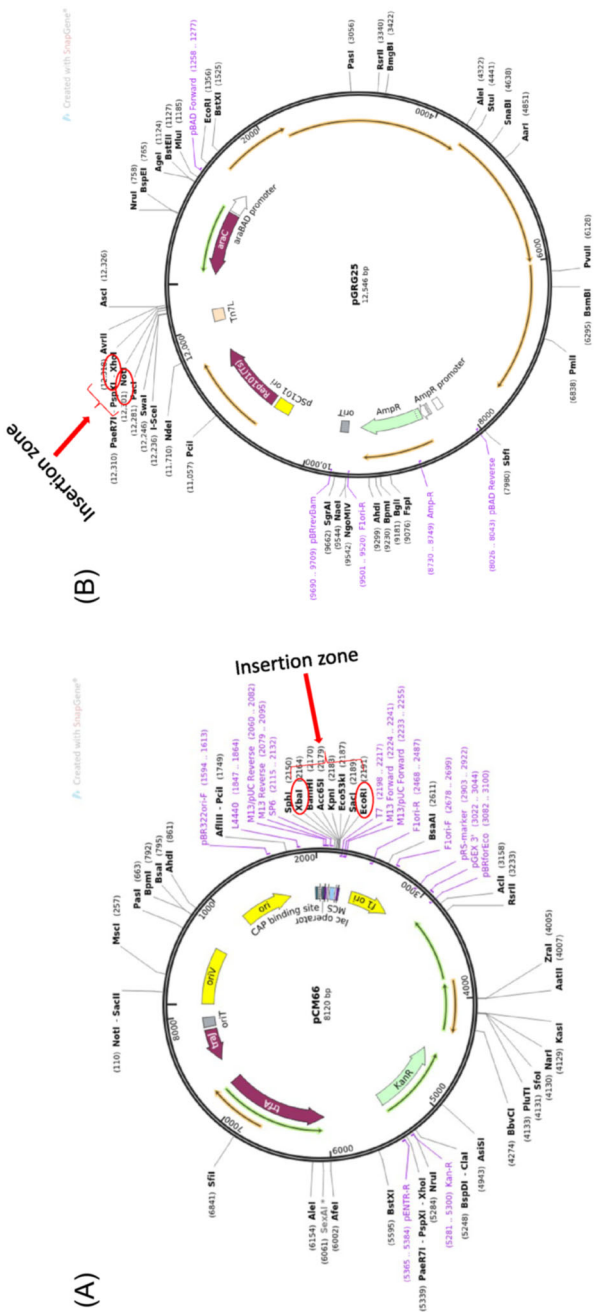

Fig. 1 Vector plasmid used for the genetic modification of *Sodalis glossinidius* (A: pGRG25; B: pCM66).

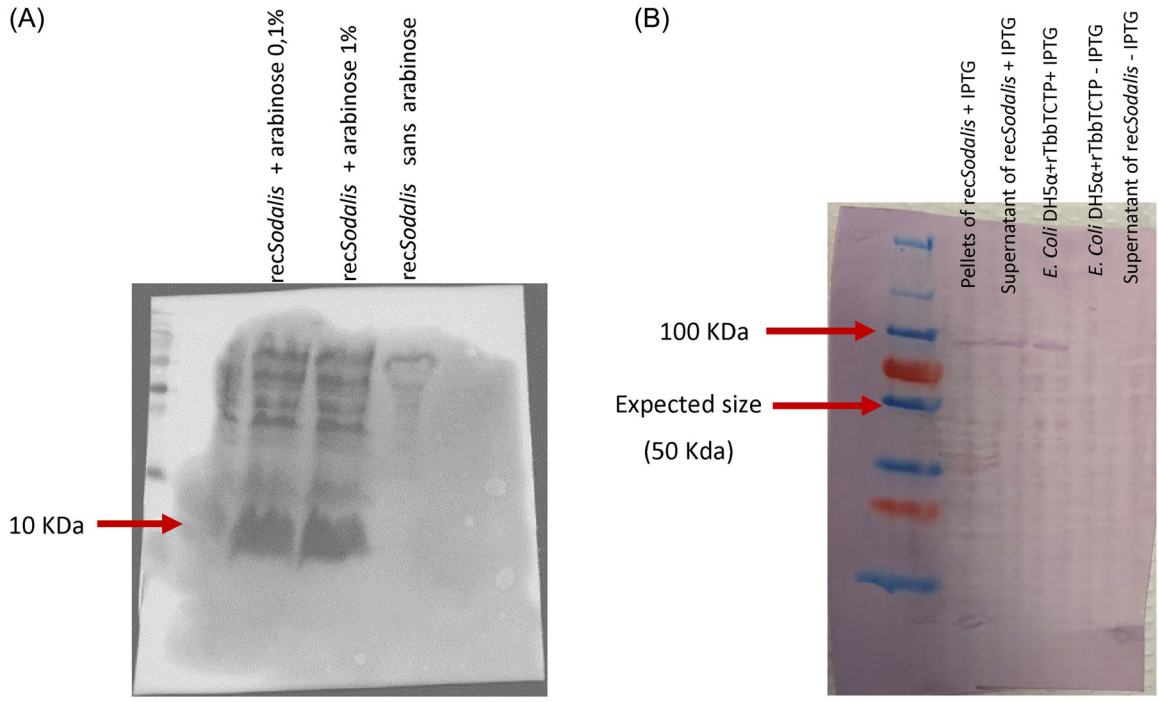

Fig. 2 Western blots showing the production of either *Tbg*TCTP (A) or cecropin (B) assessing the correct functioning of the corresponding transgenes.

tein was twice that expected, despite being recognized by the specific anti-*Tbg*TCTP antibody used.

Due to its low molecular weight (5 kDa), cecropin separation on polyacrylamide gels proved challenging, even with high concentration (20%) gels, resulting in diffuse bands (Fig. 2A). However, a clear difference was observed in recombinant *Sodalis* extracts from cultures supplemented with arabinose (inducer of transgene expression) compared to control (not supplemented with arabinose), indicating cecropin transgene expression.

Recombinant symbiont microinjection into third-instar larvae and transgenes stability

Initial microinjection targeting in utero larvae via dissected abdomens of pregnant tsetse flies were inconclusive (data not shown), likely due to challenges in accurately determining the larval stage. Subsequently, microinjections-10^6 CFU of rec*Sodalis* were therefore carried out on third-instar larvae immediately after the larviposition and prior pupation. Since the microinjection of tsetse was not performed altogether on the same day, to ensure transgene stability throughout the experiment, we monitored the presence of *cecropin*- and *TbgTCTP*-encoding genes during the bacterial culturing process. This verification confirmed stable gene retention even after 20 bacterial generations. Briefly, we checked for the presence of recombinant genes each time the recombinant *Sodalis* were resuspended in fresh culture medium, repeated over 20 generations.

Effect of recombinant symbiont microinjection into larvae on fly emergence

Two sets of experiments were carried out separately, one with rec*SodalisCec* (*cecropin*) and the other with rec*SodalisTbgTCTP*. Each experiment included larvae receiving an injection of rec*Sodalis* (*TbgTCTP* or *Cec*), larvae receiving only sterile water (to control the possible physical effect of microinjection on fly emergence) and untreated larvae (general control).

Untreated pupae exhibited a high emergence rate (95.8%, $n = 115$). Microinjection of *cecropin* (C_Cec_MI-H_2O) significantly reduced emergence (50.7%, $n = 104$; P-value $= 0.004$) compared to control without injection. Microinjection of TCTP (C_TCTP_MI-H_2O) also resulted in a lower emergence rate (70%, $n = 220$) than the control without injection, although this difference was not statistically significant (P-value $= 0.171$) (Table 2, Fig. S1). Emergence rates

Table 2 Effect of larval treatments on fly emergence.

Experimental design	Number of larvae	Number of emerged flies (*n*) (%)	*P*-value
Control_ØMI	120	115 (95.83%)	
Control_TCTP_MI-H_2O	314	220 (70.06%)	
Injected_with_r*TbgTCTP*	581	39 (6.71%)	< 0.0001
Control_Cec_MI-H_2O	205	104 (50.73%)	
Injected_with_*cecropin*	410	209 (50.97%)	> 0.9999

Control_ØMI = control without microinjection; Control_TCTP_MI-H_2O = control TCTP microinjected with H_2O; Control_Cec_MI-H_2O = control cecropin microinjected with H_2O; (%) = percentage.

Table 3 Verification of the content of pupae, injected with either H_2O or rec*Sodalis* expressing *TbgTCTP*, from which no live fly emerged.

Category of content	Pupae H_2O injected (*n*) (%)	Pupae r*TbgTCTP* injected (*n*) (%)
Empty pupae	29 (42.0%)	125 (48.6%)
Rotting pupae	13 (18.8%)	41 (15.9%)
Neoformed clear	13 (18.8%)	32 (12.4%)
Neoformed dark	10 (14.5%)	29 (11.3%)
Formed but dead flies	4 (5.8%)	30 (11.7%)
TOTAL dissected pupae	69	257

between control groups (H_2O larvae injection) across both experiments showed slight variation despite identical procedure (*cecropin* control 50.7% vs. *TCTP* control 70%), but this difference was not statistically significant (*P*-value = 0.166) (Fig. S1).

In the cecropin assay, emergence rate was statically comparable (*P*-value > 0.999) between water-injected larvae (50.73%, *n* = 104) and recombinant *Sodalis*-injected larvae (50.97%, *n* = 209). Conversely, *TCTP* microinjection significantly reduced emergence (6.71%, *n* = 581) compared to the control microinjected with water (70.06%, *n* = 220) with a highly significant *P*-value (*P* < 0.001).

To search for the reason of this low emergence rate, 4 d post-emergence of the last fly, respectively, 69 water microinjected pupae and 257 rec*SodalisTbgTCTP* microinjected pupae that had not emerged were randomly selected and dissected to verify their content. Dissected pupae were classified into five categories: empty pupae, rotting pupae, clear neoformed flies, dark neoformed flies, and dead flies (flies that were completely formed but unable to emerge). The Table 3 shows the number and percentage of pupae in each category. According to the unpaired *t*-test, there is no significant difference (*P*-value = 0.086) between rec*SodalisTbgTCTP* injected pupae and water control pupae, whatever the category.

Effect of recombinant symbiont microinjection into larvae on the pupae weight

In addition to the emergence rates, we compared pupae weight before (D0) and 2 d (D2) after larvae microinjection (either with water or with rec*CecSodalis*; here only the experiments involving cecropin was conducted) (Table S1). Since the emergence rates from larvae microinjected with water or rec*CecSodalis* were almost similar (50.73% vs. 50.97%, *P*-value > 0.999), we first compared the overall water + rec*Sodalis* microinjected pupae at D2 to the overall controls at D0, before injection. The statistical analysis (Student's *t* test) showed that the weight differences were not significant (*P*-value = 0.086). When we compared separately data from the water control group (D2 vs. D0; *n* = 90) and from the rec*CecSodalis* injected group (D2 vs. D0; *n* = 173), no statistically significant weight differences were observed whatever the group (H_2O control: *P*-value = 0.38; rec*CecSodalis* injected: *P*-value = 0.13) (Table 4).

Parasite establishment in adult tsetse flies microinjected at larval stage: checking for the presence of the recombinant symbionts in adult Glossina flies emerged from microinjected larvae

Twenty days post-infective feed, PCR analysis of dissected gut tissue from flies confirmed the presence of the corresponding transgenes in a significant proportion of individuals recombinant *Sodalis* expressing the cecropin was detected in 57.14% of emerged flies (*n* = 126), while recombinant *Sodalis* expressing *TbgTCTP* was identified in 54.16% of emerged flies (*n* = 24). The results suggest a similar establishment rate of the recombinant *S.*

Table 4 Weight comparison of pupae before injection of either H_2O or rec*CecSodalis*, and 2 d after the third-instar larvae microinjection.

	Control_Cec_MI-H_2O ($n = 90$)		Injected with rec*CecSodalis* ($n = 173$)		Overall ($n = 263$)	
Group	Weight before (g)	Weight after (g)	Weight before (g)	Weight after (g)	Weight before (g)	Weight after (g)
Mean	0.023638	0.023297	0.022874	0.022412	0.023135	0.022714
SD	0.002560	0.002644	0.002929	0.002827	0.002827	0.002792
SEM	0.000270	0.000279	0.000223	0.000215	0.000174	0.000172
P value	0.3804		0.1361		0.0864	

Control_Cec_MI-H_2O = control cecropin microinjected with H_2O; SD = Standard Deviation, SEM = Standard Error of the Mean.

Table 5 Emergence rates of *Glossina* flies that harbor rec*Sodalis* expressing either *cecropin* or *TbgTCTP* (estimated by Polymerase Chain Reaction, PCR).

	cecropin	*TbgTCTP*
Glossina flies that have emerged	209	39
Number of dissected flies	126	24
Glossina flies hosting the rec*Sodalis* (*n*) (%)	72 (57.14%)	13 (54.16%)

glossinidius strains regardless of the transgene, with no significant difference observed between cecropin and *TbgTCTP* groups (Fisher' exact test, *P*-value = 0.906) (Table 5).

Effect of recombinant proteins on the establishment of the trypanosomes in the gut of tsetse flies

Microscopic examination and PCR analysis were performed on dissected guts from tsetse flies harboring the recombinant *Sodalis* strains (rec*CecSodalis*, $n = 72$; rec*SodalisTbgTCTP*, $n = 13$) alongside various control groups (C_Cec_MI-H_2O, $n = 85$; C_TCTP_MI-H_2O, $n = 156$; and C_ØMI, $n = 113$) (Table 6). Statistical analysis revealed a significant difference ($\chi^2 = 17.296$; df = 4; *P*-value = 0.002) in parasite distribution between microinjected and non-microinjected flies.

Further analysis with the Kruskal–Wallis test identified a specific difference ($\chi^2 = 12.177$, df = 2, *P*-value = 0.001) among the control groups (C_Cec_MI-H_2O, C_TCTP_MI-H_2O and C_ØMI). This suggests a potential underlying effect of microinjection itself on parasite presence.

Interestingly, while the rate of the parasite infection in tsetse flies microinjected with rec*CecSodalis* was numerically lower compared to controls (C_Cec_MI-H_2O and C_ØMI), this difference did not reach statistical significance (Kruskal–Wallis: $\chi^2 = 0.27472$, df = 2, *P*-value = 0.872). The limited sample size of flies carrying rec*SodalisTbgTCTP* precluded statistical analysis for this group.

Table 6 Infection rate of *Trypanosoma brucei gambiense* in *G. p. gambiensis* 21 d after infection.

	Glossina flies hosting the rec*Sodalis*-cecropin	C_Cec_MI-H_2O *Glossina* flies	*Glossina* flies hosting the rec*Sodalis-TCTP*	C_TCTP_MI-H_2O *Glossina* flies	Control *Glossina* flies (Without microinjection)
Total dissected flies (*n*)	72	85	13	156	113
Number of infected *Glossina* (by microscopic observation)	6 (8.33%)	9 (10.58%)	3 (23.07%)	37 (23.71%)	10 (8.88%)
Number of infected *Glossina* (by Polymerase Chain Reaction)	–	31 (36.47%)	4 (30.77%)	52 (33.33)	52 (46.02%)

C_TCTP_MI-H_2O = control Tumor Control Translational Protein (TCTP) microinjected with H_2O; C_Cec_MI-H_2O = control cecropin microinjected with H_2O; (%) = percentage.

Discussion

Genetic engineering applied to arthropod vectors has been reported as a tool that can be used as a complementary approach to control vector-borne diseases by blocking the establishment of the parasite in its vector (Beard *et al.*, 1993). In this context, a para-transgenic approach that involves recombinant *S. glossinidius*, the tsetse fly secondary symbiont, has been investigated to deliver antiparasitic compounds into the fly's gut as a tool to make the fly refractory to trypanosome infection, thus controlling parasite transmission. *S. glossinidius* is metabolically dependent on the tsetse fly and maternally transmitted to the offspring. Consequently, it would ensure resistance inheritance and prevent horizontal transfer of the transgene. To date, trials using this symbiont as vector for the expression of foreign protein have been done in the context of the acute form of trypanosomiasis due to *T. brucei rhodesiense* transmitted in East Africa by *G. m. morsitans* (Cheng & Aksoy, 1999; De Vooght *et al.*, 2012; De Vooght *et al.*, 2014; De Vooght *et al.*, 2018; Yang *et al.*, 2022).

The aim of this study was to evaluate the effect of two compounds, an antimicrobial peptide, cecropin protein, and TCTP, a tumor protein secreted by the parasite, on the establishment of *T. b. gambiense* into the gastrointestinal tract of the vector, *G. p. gambiensis*. To achieve this, we used a para-transgenic approach, by developing functional recombinant strains of *S. glossinidius* that express the target proteins *in situ*.

This study successfully established recombinant *S. glossinidius* strains harboring either the pCM66*TbgTCTP* (encoding *TCTP*) or the pGRG25*Cec* (encoding *cecropin*) plasmids. These first steps, crucial for the following experiment, were verified by western blot analysis, confirming transgene expression, and by Sanger sequencing, demonstrating integration into the plasmid. Furthermore, we successfully reimplanted these recombinant bacteria into tsetse flies at the larval stage. However, establishing these recombinant strains also presented some challenges.

Detection of the recombinant cecropin protein presented limitations. Colorimetric detection was likely unsuccessful due to insufficient sensitivity and potentially low plasmid numbers, as observed by McKenzie & Craig (2006). Additionally, protein transfer from gel to membrane proved challenging, similar to the experience of Durvasula *et al.* (1997).

In contrast to colorimetric technique, western blot analysis using chemiluminescence revealed a strong, yet diffuse, band near the expected size of cecropin. However, several additional, higher molecular weight bands were also observed. Since Ni-NTA purification, employed by De Vooght *et al.* (2012), was not performed in our study, and it is known that a large range of proteins contain number of histidine residues, the chemiluminescent detection may be overly sensitive. Further purification of His-tagged cecropin via affinity chromatography (Ni-NTA) is warranted.

Alternatively, these additional bands could represent protein complexes formed by multiple cecropin unit or interaction with others. However, this is debatable because: (i) sample preparation included dithiothreitol (DTT), which disrupts disulfide bridges potentially linking cecropin molecules; (ii) the additional proteins were only observed in rec*Sodalis* culture supplemented with arabinose, the transgene expression inducer. Therefore, additional proteins are likely "associated" with cecropin expression. Future studies should include Ni-NTA purification step in order to enhance specificity and get a clean result.

Regarding TCTP, the expression of the *Tbg*TCTP was verified by western blot with a specific antibody. Only one protein band was shown, but displaying 100 kDa molecular weight instead of the expected 50 kDa. It could be the result of a dimerization of the produced *Tbg*TCTP despite the presence of DTT when preparing the samples to be analyzed.

Mechanical microinjection of water (control) or recombinant *Sodalis* expressing either *cecropin* or *TbgTCTP* reduced fly emergence compared to the non-microinjected larvae (∼30% to 50% decrease): this is principally due to the level of wound of the larvae. Interestingly, *cecropin* expression in recombinant *Sodalis* did not further impact the emergence rate compared to the control. Conversely, microinjection of *TbgTCTP* resulted in a drastic reduction in the emergence rate (6.71%).

Our observation of a significantly lower emergence rate in the flies injected with *TbgTCTP* suggests a potential disruption of normal larvae development. This finding aligns with the known multifunctional nature of the TCTP in various organisms, encompassing roles in cell proliferation (Stierum *et al.*, 2003), stress responses (Brioudes *et al.*, 2010) embryo development (Roque *et al.*, 2016) and apoptosis (Susini *et al.*, 2008). *Tbg*TCTP might be triggering apoptosis or other detrimental process within the developing larvae.

Furthermore, Bossard *et al.* (2017) demonstrated that TCTP can modulate the *in vitro* growth of bacteria isolated from the tsetse fly microbiome. This raises an intriguing possibility: does *Tbg*TCTP expressed by the injected recombinant *Sodalis* disrupt the fly's microbiome composition *in vivo*? Such a disruption could indirectly interfere with larval development and contribute to the

observed mortality. Future studies investigating the impact of *Tbg*TCTP on larval development and microbiome composition are warranted to elucidate the underlying mechanisms.

Our study explored the potential antiparasitic effect of cecropin and *Tbg*TCTP in *S. glossinidius* infected with *T. b. gambiense*. While a slight decrease in infection rate was observed in flies harboring cecropin-expressing bacteria, this difference was not statistically significant. Unfortunately, the low emergence rate from *TbgTCTP*-treated larvae prevent further analysis of its potential effect. These findings did not show a clear correlation between the cecropin expression and the absence of the parasite like the previous studies of Durvasula *et al.* (1997) who demonstrated a clear correlation between cecropin production and the absence of *T. cruzi* in triatome bugs; that could be due to the difference of parasite species, even if they belong to the same genus. Interestingly, in our own experiment, the overall infection rate was lower than observed in control groups, though not statistically significant. This suggests a potential suppressive effect of cecropin as observed for *T. cruzi* in triatomes (Durvasula *et al.*, 1997) or for *Plasmodium* oocysts in *Anopheles gambiae* (Kim *et al.*, 2004).

Although our results do not allow to conclude on the effect on fly infection of cecropin or TCTP delivery by para-transgenesis into the fly's gut, they are nevertheless encouraging insofar as the technical approach is working on *G. p. gambiensis*. Further work is needed to verify whether cecropin and/or TCTP are really effective in reducing the infection rate of tsetse flies. Similarly, identification of excreted secreted proteins (ESPs) by the trypanosome (Atyame Nten *et al.*, 2010; Geiger *et al.*, 2010), as well as differentially expressed genes (DEPs) associated with the susceptibility/resistance of the fly to trypanosome infection (Hamidou Soumana *et al.*, 2015) should enable new targets to be selected and tested in the future.

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Author contribution

Anne Geiger and David Berthier-Teyssedre conceived the experiments. François Sougal Ngambia Freitas, Linda De Vooght, Flobert Njiokou, Jan Van Den Abeele, Géraldine Bossard, Rosa Milagros Corrales, Sophie Ravel, Bernadette Tchicaya and David Berthier-Teyssedre performed the experiments. François Sougal Ngambia Freitas, Linda De Vooght, Jan Van Den Abeele, David Berthier-Teyssedre and Anne Geiger analyzed the data. Jan Van Den Abeele and Anne Geiger contributed reagents/materials/analysis tools. François Sougal Ngambia Freitas, Linda De Vooght, Flobert Njiokou, Jan Van Den Abeele, David Berthier-Teyssedre and Anne Geiger wrote the paper.

Disclosure

The authors declare that they have no competing interests.

Ethics statement

The experiments designed for this study were approved by the Regional Ethic Committee for Animal Experimentation CEEA-LR 36 under project number APAFIS#13264-2018012915201897 and authorized by MENESR (Ministère l'Education Nationale de l'Enseignement Supérieur et de la Recherche).

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 (A) Comparison of emergence rates between microinjected flies with water compared to control without microinjection. (B, C) Comparison of emergence rates between microinjected groups.

Table S1 Weight of pupae before injection of either H_2O or recCec*Sodalis*, and 2 d after the third-instar larvae microinjection.