Comparative genomics and transcriptomics of the *Spiroplasma glossinidia* strain *s*Gff reveal insights into host interaction and trypanosome resistance in *Glossina fuscipes fuscipes*

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Supplementary Document 1

Supplementary Document 1: detailed protocol

Adapted from Masson, F., Calderon Copete, S., Schüpfer, F., Garcia-Arraez, G., Lemaitre, B., 2018. *In Vitro* Culture of the Insect Endosymbiont *Spiroplasma poulsonii* Highlights Bacterial Genes Involved in Host-Symbiont Interaction. mBio 9, e00024-18. https://doi.org/10.1128/mBio.00024-18

Preparation of fly extract

- Collect 6 g flies (*Gff*) aged 10-24 days, starved for 2 days
- Crush with sterile mortar and pestle in BSK-H, 30 ml per 6 g flies
- Incubate 20 min at 56 °C, let cool to room temperature
- Centrifuge 15' at 4000 rpm
- Centrifuge 15' at 13200 rpm
- Filter at 0.45 µM
- Filter at 0.22 µM
- Aliquot and store at -20 °C

Components of lipid mix

Cholesterol	10 mg
Palmitic acid	5 mg
Sphingomyelin	10 mg
Ethanol absolute warmed to 30 °C	1.8 mL
1-Palmitoyl-2-oleoyl-sn-glycerol (10 mg/ml in Ethanol)	100 µl
1,2-dioleoyl-sn-glycerol (20 mg/ml in Ethanol)	100 µl
Tween 40	50 µl
Tween 80	50 µl
Oleic acid	5.6 µl

Prepare 6% BSA stock (fatty acid free) and mix 19.6 mL BSA 6% + 400 µl lipids

BSK-H preculture medium: 33.6 mL

BSK-H medium without L-glutamine (Bio&Sell)	32 mL
Fly extract in BSK-H	1.6 mL

BSK-H-spiro medium: 50 mL

BSK-H medium without L-glutamine (Bio&Sell)	39.25 mL
Penicillin	0.25 mL
Arginine	1.25 mL
Rabbit Serum	3 mL
Fly extract in BSK-H	3.75 mL
Lipids mix	2.5 mL

Adjust pH to 7.5 and filter at 0.22 µm. Store at 4 °C for up to 1 week.

Protocol: Initiation of *s*Gff *in vitro* culture

Establishment of the *s*Gff *in vitro* culture followed the protocol of [1], developed and optimized for the cultivation of *Spiroplasma poulsonii* MSRO. This protocol is based on Barbour-Stoenner-Kelly H (BSK-H) media without L-Glutamine (Bio&Sell, Germany), originally designed for *Borrelia burgdorferi.* The method comprises of a precultivation step in BSK-H with fly extract before inoculation in complete BSK-H-spiro media supplemented with rabbit serum, fly extract, lipids, antibiotics and amino acids. Culture inoculation and maintenance steps were all performed under sterile conditions.

The *Spiroplasma*-infected *Gff* line used for the initiation of the preculture was selected for high *Spiroplasma* infection prevalence, as described in [2]. Hemolymph was extracted from 10 females and 10 males aged seven to 21 days by surface-sterilization, removal of one prothoracic leg and aspiration of the exposed hemolymph droplet using a 10 µl pipette tip. Precultures were initiated in three biological replicates by inoculating 3.2 ml of preculture medium (BSK-H with 5% filtered fly extract) with 10 µl hemolymph and incubated at 25°C for 14 days without agitation under microaerobic atmospheric conditions using the CampyGen™ Gas-Pak system (ThermoFisher Scientific) to maintain an atmosphere of 8-9% O_2 and 7-8% CO_2. The presence of *s*Gff was validated by fluorescent microscopy using a Leica DMi8 inverted fluorescent microscope (Leica Microsystems) following staining with SYTO 9 (0.025 mM;ThermoFisher Scientific). *Spiroplasma* presence was further confirmed by PCR-amplification using *Spiroplasma*-specific 16s rRNA primers (Supplementary Table 1).

Precultures were pelleted by centrifugation for 40 min at 2000g, resuspended in 3.2 ml of BSK-H-spiro media (BSK-H + 7.5% fly extract, 6% rabbit serum, 0.5% Penicillin, 2.5% arginine, 5% lipid mix) and aliquots of 100 µl stored at -80°C. Cultures were prepared using 100 µl frozen preculture aliquots in 3.2 ml of BSK-H-spiro media at 25°C in microaerobic conditions without agitation for the establishment of active long-term cultures and cultures for growth curve analysis. Cultures underwent routine microscopy checks every 10-14 days to validate presence of *s*Gff and to assess bacterial density before passaging. Passaging of bacteria was done every 10-14 days by diluting the culture 1:1 with freshly prepared BSK-H-spiro media. Every fourth passage until at least passage 12, bacteria were pelleted at 2000 x g for 40 minutes and the media were completely renewed.

References

1. Masson F, Calderon Copete S, Schüpfer F, Garcia-Arraez G, Lemaitre B. *In Vitro* Culture of the Insect Endosymbiont *Spiroplasma poulsonii* Highlights Bacterial Genes Involved in Host-Symbiont Interaction. mBio. 2018;9:e00024-18.

2. Dera KM, Barro DT, Kaboré BA, Gstöttenmayer F, Dieng MM, Pagabeleguem S, et al. *Spiroplasma* infection in colonized *Glossina fuscipes fuscipes*: impact on mass rearing and the sterile insect technique. Insect Sci. 2025;1744-7917.70078.