Identification of Sialidase-Producing Bacteria in the Tsetse Gut and Characterisation of a Paenibacillus Sialidase: A Potential Tool for Paratransgenic Control of African Trypanosomes

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SUPPLEMENTARY INFORMATION

1. Introduction

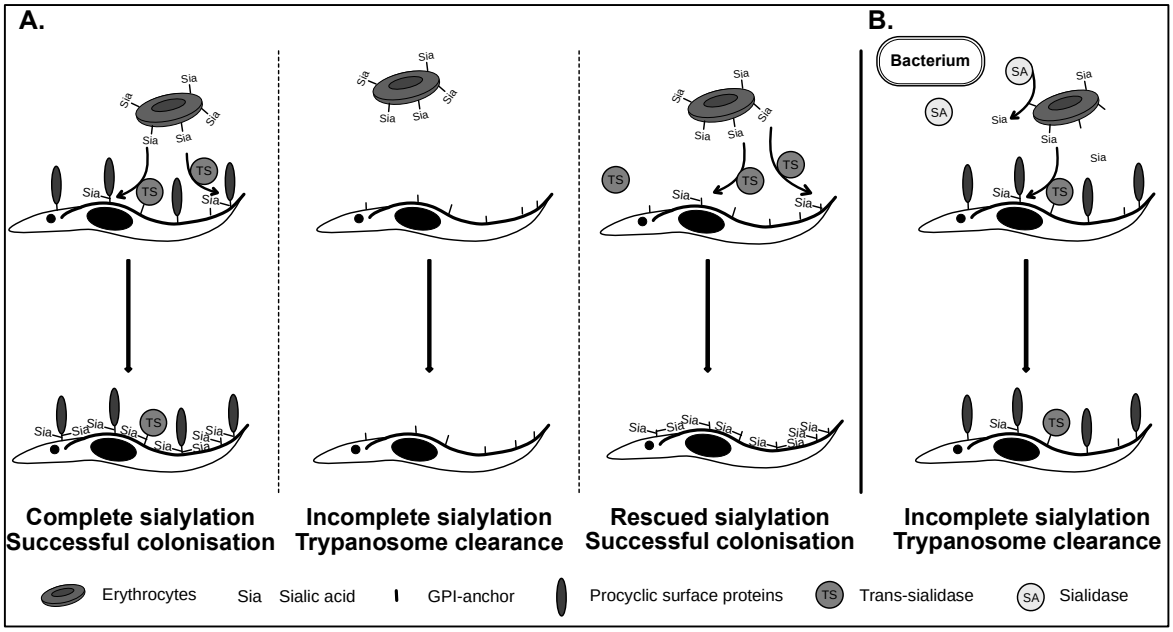


Figure S1: Model: surface Sia coat protects the tryponsomes in the tsetse midgut. A. Simplified representation of the work from Nagamune *et al*. [1]. GPI anchors on the surface of procyclic trypanosomes are sialylated. When TS is not present, colonisation efficiency is severely impaired. Expression of soluble TS rescues infection efficiency. **B**. Hypothesis: Sialidase expressing bacteria in the tsetse gut impair trypanosome colonisation. Sialidase and TS compete for the same substrates, released SA cannot be transferred by TS. By substrate depletion and enhancing the concentration of acceptor glycans, bacterial Sialidase activity results in incomplete sialylation of the parasites leading to clearing of the parasites.

2. Supplementary Materials and Methods

2.1. Dissection of tsetse flies

Only life flies were dissected and analysed. They were dissected under normal atmospheric conditions in a self-constructed mobile field cabinet creating a semi-sterile work environment. The inside of the cabinet, dissecting tools and microscope slides were thoroughly cleaned with 70% ethanol and air-dried. Flies were dissected using microsurgery magnifying glasses on paper towels previously soaked with 70 % ethanol. Wings were removed and stored dry. The remaining flies were dipped in 70 % ethanol for 10 s to sterilise the surface and allowed to air-dry prior dissection. The dry fly was then dissected in sterile PBS on a clean slide. Legs, proboscis, salivary glands were collected and preserved in 200 µL nucleic acid preservation agent (NAPA: 25 mM sodium citrate, 10 mM EDTA, 70 g ammonium sulfate/100 mL solution, pH 7.5) in 1.5 mL cryotubes. Gut was homogenised in sterile 50 mM Tris buffer pH 9.0 using a motor-driven pestle (Kimble Kontes) in a microfuge tube. Gut homogenate was distributed between five different assays.

2.2. On-field sialidase assay optimisation

Our goal was to detect sialidase activity reliably in single tsetse gut samples collected in the field. Therefore, the corresponding assays had to be adapted to field conditions, i.e. no laboratory infrastructure available during field collections, no or unreliable electricity, difficult logistics and very limited cooling opportunities. For the purpose of the study, it was essential to see whether (1) we can identify active sialidase in the fly gut, (2) whether free SA are initially present in these samples resulting from a recent blood meal and (3) whether SA are stable in the fly gut during assay conditions, including enzymatic degradation of SA by SA-lyase. Therefore, the initial approach of the sialidase assay included separating gut homogenate in five parts:

1) 50 µL were transferred to 450 µL of NAPA for nucleic acid analysis.
2) 40 µL homogenate was incubated with 80 µg fetuin (0.8 – 0.9 mM fetuin-bound SA) and 1 mM 4-Methylumbelliferyl-*N*-acetylneuraminic acid (MU-NANA) as substrate to detect sialidase activity.
3) To see whether free SA are initially present in these samples resulting from a recent blood meal, 40 µL of the gut homogenate was directly precipitated with 4-fold volume of acetone (no substrate control).

4) 40 µL of gut homogenate was incubated with free SA to monitor the stability of SA in the fly gut during assay conditions, including potential enzymatic degradation by sialic acid lyase (SA-lyase).
5) 30 µL of homogenate was added to 150 µL sterile MMI (Maramorosch and Mitsuhashi Insect medium) buffered with 10 mM sodium phosphate (pH 7.0) for bacterial culture.

Qualitative assessment of sialidase activity in field samples was performed using a simple filter spot approach. 1 µL of the acetone supernatant (10x diluted original sialidase assay) was spotted onto a Whatman filter. MU-fluorescence was visualised at pH 9.0/10.0 [2] by adding 2 µl of 1 M glycine stock solution (pH 10) and exposing to blue light (395-400 nm). The signal was visualised with a commonly available blue light torch (Wavelength 395-400 nm). Positive samples were selected for bacterial isolation.

2.3. Detection of cleaved methylumbelliferone via an anion-exchanger membrane

Supernatants of the first survey were separated on Sartobind Q 96-well stripes (Sartorius stedim biotech, Göttingen, Germany) in a 96-well format. Briefly, the anion exchange membranes were equilibrated with 5 mM Tris-Cl pH 9.0. 200 µL of assay supernatant was applied to each membrane and the flow through collected by centrifugation for 1 min at 4°C and 1000 rpm. The membranes were washed with 500 µL 5 mM Tris-Cl pH 9.0, and bound substances eluted with 500 µL 1 M NaCl. Elute was kept and frozen at -80°C. 100 µL flow through was added in a black 96-well plate for fluorescence detection of MU released. Samples of all subsequent surveys were directly investigated for fluorescence without prior anion exchange separation and 50 µL of dissolved assay supernatants were used.

2.4. Detection of free sialic acid by RP-HPLC

α-Keto acids react specifically with phenyldiamides to form quinoxalines. The α-keto function of SA has been utilised to develop a sensitive and specific detection of the acidic sugars by reverse phase high pressure liquid chromatography (RP-HPLC) after derivatization with 1,2-diamino-4,5-methylendioxybenzene (DMB) [3,4]. A modified protocol used 4,5-dimethylbenzene-1,2-diamine (DMBA), a cost-efficient derivative of DMB [5]. Sialic acid-derivatives can be separated via their hydrophobic properties provided by DMB or DMBA and quantitatively detected via their fluorescence. Concentrations of free SA in fly samples were determined by adaptation of the protocol from Hara *et. al.* [6] and Wang *et al.* [5], respectively.

For DMB derivatization, 30 µL of fly sample supernatant was mixed with 4 M acetic acid to obtain a final concentration of 2 M acetic acid. The DMB-solution (1.4 M acetic acid, 18 mM sodium hydrogensulfite, 0.75 M β-Mercaptoethanol and 7 mM DMB [6]) was prepared freshly prior each derivatization. The fly sample in 2M acetic acid and the DMB solution were mixed in a volume ratio of 1:1 and derivatization was carried for 2.5 h at 50 °C in the dark. Following derivatization, samples were spun down and the reaction slowed by keeping the samples at -20 °C for at least 15 minutes or until measured. The samples were transferred to 200 µL conical HPLC reaction tubes and kept in the dark until RP-HPLC analysis.

DMBA was dissolved in 1.2 M acetic acid to reach a final concentration of 300 mM. This solution was stable and used for experiments for up to 4 months when stored at -20°C. For derivatization, 52.8 µL assay sample was mixed with 7.2 µL 10 M acetic acid. 60 µL 6 mM DMBA solution was added and derivatization was promoted by incubation at 60°C for 60 min in a water bath in the dark. After 60 min, samples were briefly centrifuged, cooled down at -20°C to slow the reaction and either directly measured or stored at -20°C until analysed.

For both methods, a calibration curve was prepared for every measurement. A time dependent increase of fluorescence signal was observed for SA-DMB and SA-DMBA derivatives of the same concentrations measured at different time points Therefore, technical replicates of each calibration concentration were measured at the beginning, middle and end of each set of samples.

Samples were analysed at a MERCK/Hitachi HPLC system. Sialic acid-DMB(A) derivatives were separated on a reverse-phase column (Phenomenex®Luna C18(2), Phenomenex, Germany) and detected using a fluorescence detector (F-1000) at excitation wavelength 370 nm and emission 450 nm for SA-DMB and 380 nm and 430 nm for SA-DMBA. The column chamber was constantly kept at 25°C. SA-DMB and SA-DMBA derivatives were eluted isocratically at a flow rate of 1 mL/min. Sialic acid-DMB derivatives were eluted for 9 min with 7 % methanol/ 9% acetonitrile in water, followed by a 3 min wash with 7% methanol and 80% acetonitrile in water and 17 in re-equilibration. Sialic acid DMBA-derivatives were eluted for 5 min with 20% acetonitrile in water, followed by 4 min wash with 2% tetrahydrofurane in acetonitrile and 6 min re-equilibration.

The software HSM D700 was used for data acquisition and evaluation. Chromatograms were adjusted manually to allow baseline correction prior to calculation of peak areas.

Calculation of free sialic acid from RP-HPLC data

The peak area of SA-DMB(A) derivatives increased dependent on the time the samples were kept at room temperature prior to injection into the RP-HPLC system. The technical replicates of the calibration curve were used to monitor and correct for the time-dependent signal increase of the samples. In principal, for each time point a sample was analysed, a corresponding expected calibration curve was calculated to determine the concentration by interpolation of this calibration curve. Due to the differences in SA-DMB and SA-DMBA analysis, two different methods were employed for correction.

In detail, peak areas of SA-DMB derivatives were corrected by employing the slope of time dependent increase of the respective calibration concentration. The different peak areas measured in the technical replicates of one concentration were plotted against the timepoints at which they were measured. The increase of the peak areas followed a linear regression. The slopes of the regression curves were used to correct the unknown samples. The slope that represented the concentration range of the unknown sample was multiplied by the timepoint at which the sample was measured. This factor was subtracted from the measured peak area. Concentrations of the samples were calculated from the corrected area using the linear regression curve of the first calibration set.

A similar approach was adopted to correct peak areas of SA-DMBA derivatives. The peak areas within the technical replicas of the calibration curve increased in the same proportion independent of the concentration. Therefore, the quotients of the peak areas measured last (A_E) and the peak area measured first (A_1) of the respective concentrations were averaged to obtain factor F.

$$F = \frac{1}{n} * \sum_{i=1}^{n} \frac{A_{Ei}}{A_{1i}}$$

with

i sample index

F was put in relation to the time interval between two measurements (T_E-T_1). (T_E-T_1) is constant for all samples of the calibration curve, all other samples were measured within this time interval. The measured peak areas (A_S) were corrected as follows:

$$A_{SC} = A_S * \frac{(T_E - T_1)}{(T_E - T_1) + (F - 1) * T_S}$$

with

A_{SC} Peak area of sample corrected for time dependent signal increase

A_S Peak area of sample measured

(T_E-T_1) Time interval between 1st and last calibration

T_E Timepoint of last calibration

T_1 Timepoint of 1st calibration

T_S Timepoint of sample measured

Concentrations of the samples were calculated from the corrected peak areas (A_{SC}) using the linear regression curve of the first calibration set.

2.5. Sialidase assay of liquid bacterial cultures

Whole bacteria cultures, culture supernatants and bacteria lysates were screened for sialidase activity. To separate the supernatant from bacteria cells, 1 mL of liquid culture was centrifuged 5 min at 4000 rpm. For the lysate, the bacteria pellet was lysed by lysozyme digest with 250 µg lysozyme in 50 mM sodium phosphate buffer pH 7.3 supplemented with 4 mM EDTA and 0.1% Triton-X100 in a total volume of 200 µL for 5 min at 30 °C followed by 45 min on ice. EDTA was neutralised by adding 4 mM $CaCl_2$, and samples were centrifuged for 5 min at 14000 rpm and the supernatants collected.

The bacteria culture supernatant, whole bacteria culture and the lysate were investigated for sialidase activity by adding 1 mM MU-NANA with and without additional 4 mM $CaCl_2$. A control sample without any supplements was incubated in parallel. To determine any interference of fluorescence detection within the samples, whole bacteria cultures were additionally incubated with 100 µM MU.

All samples were incubated for 18 h at 30°C in the dark. Standard curves of MU in MMI and lysis buffer were loaded on the same plates. Fluorescence was measured after addition of 100 µL of 1 M glycine pH 10.0 in a black 96-well plates as described above with the adjustment that the plate was covered with adhesive foil to contain any residual live bacteria. To assess cleavage of MU-

NANA in a concentration dependent manner, liquid bacterial cultures were added to a 1:2 dilution series of MU-NANA (1000 µM to 16 µM available SA).

2.6. PCR analysis for presence of *Trypanosoma* sp.

DNA was purified from homogenised gut tissue in NAPA with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer instructions with following adjustments as described previously [7]: 100 µL of homogenate was used for purification and eluted with the same volume of elution buffer. DNA content was checked by spectrophotometric detection at 260 nm.

A diagnostic PCR targeting the Internal transcribed spacer 1 (ITS1) of Kinetoplastida was performed to investigate presence of trypanosomes in the gut samples [8]. Species were assigned according to the length of the PCR amplicon as described previously [8,9]. Preparation of the PCR reaction and amplification cycles were performed as described previously [9] with the adjustment of using 5 µL template in the first reaction. Amplicons were resolved on a 2 % TBE (Tris-borate EDTA buffer) agarose gel stained with Stain-G (Serva, Heidelberg, Germany) for 1 hour at 100V. GeneRuler 50bp DNA Ladder (Thermo Fisher, Dreieich, Germany) was used for molecular size estimation.

2.7. Bacterial DNA extraction and 16S rRNA amplification

DNA was crudely extracted from bacterial isolates by bead-beating with 0.75-1 mm glass beads in 500 µL sterile 10 mM TE-buffer pH 8.0 (10 mM Tris-Cl and 1 mM EDTA). Bead beating was repeated twice for 20 sec. Supernatant was transferred in 1.5 mL microtubes and heated at 95°C for 20 min to reduce potential spores within the samples. The crude extracts were stored at -20°C.

The bacterial 16S rRNA gene was amplified with generic primers according to Grönemeyer *et al.* [10]. 2 µL template were used for amplification in a final volume of 50 µL with 2.5 mU Dream*Taq* Polymerase, 5 µL Dream*Taq* Buffer and 50 µM sterile dNTPs (all Thermo Scientific, Dreieich, Germany). Primers used were Bac8uF 5'-AGAGTTTGATNHTGGYTC AG-3' and Univ1492uR 5'-GGNTCCTTGTTACGACTT-3', both at final concentrations of 0.5 µM. PCR amplification cycle was as follows: 5 min at 95°C, 40 cycles of 1 min at 95°C, 30 sec at 52 °C and 2 min at 72 °C, final elongation step for 10 min at 72°C.

Successful amplification of the approximate 1500 bp amplicon was investigated on an 1.5% TAE agarose gel prior direct sequencing by a commercial company (Microsynth SeqLab, Göttingen,

Germany). Obtained sequences were cleaned and trimmed using Geneious Pro 5.5.9 [11]. They were aligned using the Geneious Alignment tool. Sequences were blasted against the NCBI database to identify closest relatives. Reference sequences obtained from NCBI and sample sequences were aligned and a 689 bp alignment was analysed. Phylogenetic relationships were inferred with MEGA6 [12]. Maximum likelihood analysis was performed with the Kimura-2-parameter model assuming gamma distribution as determined by the MEGA model finder tool with 1200 bootstraps replications.

2.8. Purification of bacterial genomic DNA

Genomic DNA was isolated from pure bacterial culture using standard phenol-chloroform method [13] with few modifications. Briefly, stock culture was streaked onto MMI-agar-10%FCS plate and a single colony was used to inoculate 15 ml MMI-10% FCS. In two centrifugation steps, 2 x 2 ml of cells (OD_{600nm} 1.5) were centrifuged in one cup for 5 min at 4000 rpm. The pellet was resuspended in 1 mL TES-buffer, centrifuged again and the pellet finally resuspended in 300 µl TE. For cell lysis, 100 µL of 5% Laurylsarcosin and 23 µL 5% Pronase E were added, vortexed and incubated for 1 h at 37°C. Then 5 µL RNase (10 mg/mL) were added and incubated for 30 min at 37°C. The mixture was pipetted with the blue tip at least 10 times up and down and well vortexed. To the cell suspension, the same volume of PCI (phenol:chloroform:isoamylalcohol, 24:24:1 vol/vol) was added, well mixed and centrifuged for 10 min at 14,000 rpm. The upper phase was carefully transferred with a 100 µl pipette into a new Eppendorf tube without touching the interphase. The adding of PCI, mixing, centrifugation and the transfer of the upper phase was repeated for a minimum of 3 more times. Then the upper phase was finally extracted with TE-saturated chloroform-isoamylalcohol and centrifuged again. The supernatant was discarded, and the pellet was washed 1x with 70% ethanol (500 µL), centrifuged shortly and the supernatant discarded with a pipette. The residual ethanol was removed and the pellet was dried at 37 °C. Finally, the pellet was resuspended in 30 µL of TE buffer. The DNA concentration and the purity was measured on NanoDrop then stored at -20 °C. For integrity assessment, about 700 ng of DNA were run on 1% agarose.

2.9. Sialidase cloning

Amplification of the sialidase gene with restriction sites: Primers (Sigma-Aldrich) were designed to amplify the entire gene except the start and stop codons and to include BsphI and EcoRI restriction enzyme recognition sites to facilitate cloning into the protein expression vector pET28a. Primers were BsphI_His_Sia: 5'-tatcatgagccatcaccatcaccatCACATTCCATTTCG-3' and EcoRI_Sia: 5'-tcgaattcATCGCCATCATGTTCATCCTC-3' [nucleotides in lower case are extensions containing the recognition sites for restriction enzymes (underlined) and the 6X His-Tag (double underlined)]. The reaction mix was constituted of following: 1.0 µM of each of the primers, 1µL of 10 mM dNTPs, 1 unit Phusion High-Fidelity DNA Polymerase (ThermoFisher), 3 µL DNA template, 10 µl of 5X Phusion™ HF Buffer and H_2O for a total volume of 50 µL.

For amplification reaction, after optimization, 2 continuous steps PCR were performed in following conditions: an initial denaturation step at 95 °C for 3 min was followed by a first 5 amplification cycles including: a denaturation step at 94 °C for 10 s, an annealing step for 30 s at 48 °C and an extension step at 72 °C for 1 min 20 s; then followed immediately by a second 20 amplification cycles at the same conditions except the annealing temperature that was increased to 65°C. A final extension step was performed at 72 °C for 10 min.

After the PCR amplification, 5 µL of amplified products were resolved by electrophoresis at 100 volts for 60 min on 1% agarose gel stained with Stain-G to investigate the successful amplification of the 2784 bp amplicon. DNA bands were visualized under ultraviolet light. Then, the remaining reaction volume was run on TAE agarose gel. The amplicons were excised and purified using the GeneJET Gel Extraction Kit (ThermoScientific) according to the manufacturer instructions.
The purified amplicons (tagged sialidase gene) were cloned into pJET1.2/blunt cloning vector (ThermoFisher), transformed into *E. coli* strain XL1-Blue according to manufacturer instructions and sequenced to confirm successful amplification of the targeted sequence with restriction sites.
Expression plasmid: The expression plasmid used in this study was pET28a-MBP-SpeI-TS1a-SNAP-Strep. The original vector (pET28a) was already modified with insertion of other elements [14].
Restriction digestion: The amplified sialidase gene was treated with restriction enzymes: FastDigest BspHI and EcoRI (ThermoScientific), while the expression plasmid pET28a was treated with FastDigest EcoRI and NcoI. The enzyme NcoI used for the expression plasmid

produces compatible end with BspHI even though they recognize different restriction sequences. The restriction digestion was performed according to manufacturer instructions.

Ligation: The insert was ligated with expression plasmid pET28a using T4 DNA Ligase (ThermoScientific) according to manufacturer instructions. The mixture was then transformed into *E. coli* strain Rosetta and plated on LB-agar containing kanamycin. Successful and correct gene insertion was verified by colony PCR using three set of primers: the first set amplifying the T7 promoter to the T7 Terminator (3008 bp), the second amplifying from one portion of the gene to the T7 Terminator (2732 bp) and the third set targeting one portion of the sialidase gene (541 bp). The constructed plasmid was further sequenced to confirm the sequence and the directionality of the insert.

3. Supplementary results

3.1. Sialidase Field Assay optimisation

The sialidase assay was first applied in 385 tsetse during a field survey in Nigeria during March 2014 [7]. All samples were screened for sialidase activity by direct fluorescence detection of free MU. A subset of samples showing a >5-fold increase over autofluorescence measured in the sample without added substrates and a random selection of samples representing the different locations were analysed for presence of free Sia with RP-HPLC of DMB(A)-derivatives. An overview of the samples screened by both detection methods is presented in Fig. S2. Two distinct populations are identified that translate to two different challenges faced during the application of the assay in the field.

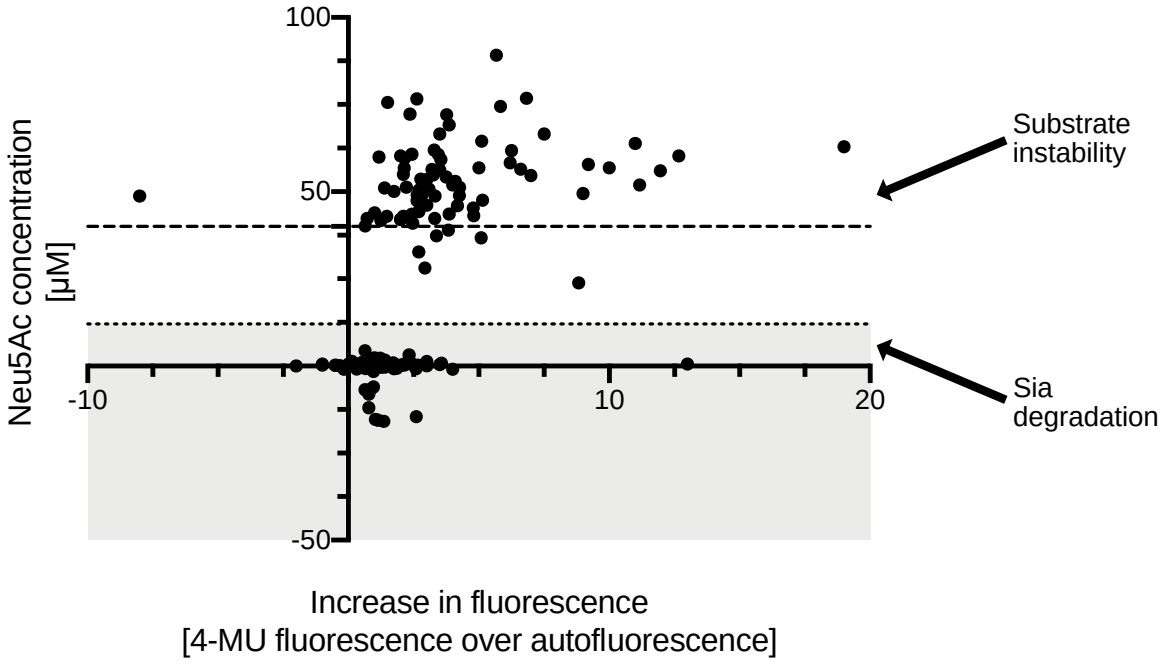


Figure S2. Sialidase assay results of the first field application in tsetse gut samples collected in Nigeria (2014). The dashed line represents free SA present in substrate mixtures used to assay all samples above they grey area. The dotted line represents the average free SA present in the substrate mixtures used with all samples clustering within the grey area. Free SA concentrations were determined in the Sialidase assay samples by analysis of DMB and DMBA derivative of SA by RP-HPLC as described under methods. Increase in fluorescence was determined by in the Sialidase assay supernatants measured after anion exchange chromatography.

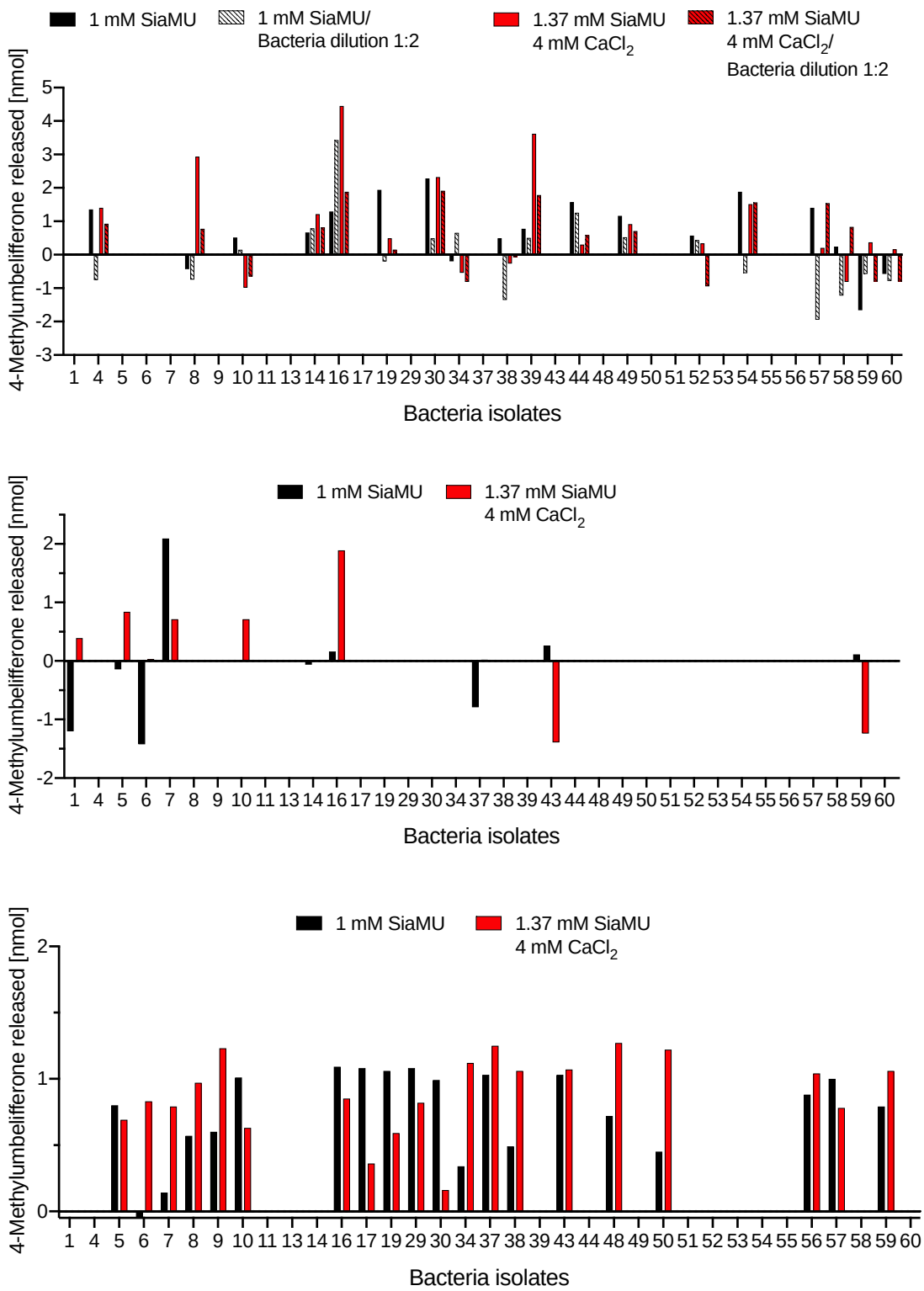
The first challenge regarded substrate stability. During field collections, fetuin and MU-NANA were incubated in buffer in parallel to the sialidase assay in fly samples to monitor stability of our substrate mixture (substrate controls). On each day of employing the sialidase assay, substrate controls were included to link irregular high concentrations of free SA to potential substrate degradation at a timepoint during collection. And indeed, substrate degradation was observed. All samples with free SA concentrations above the dotted line (Fig. S2) were dissected at the first and second collection sites of the survey. Correspondingly, the substrate control of the first site showed a high background of free SA, represented by the dashed line. Free SA concentrations in the substrate control indicate hydrolysis of the substrates during field conditions. This masks potential positive candidates. It should be noted that though temperature fluctuated extensively, ranging between 26°C and 38°C during the enzyme assays, no correlation between high temperature and substrate instability was detected. High free SA concentrations were detected in the first 50

289 samples incubated at constant 27 °C. To enhance substrate stability, lyophilised substrates were
290 used in all subsequent field screenings.

291 The second challenge identified during RP-HPLC analysis is represented by samples below the
292 dotted line. In all samples following a specimen collected and dissected during the first day of the
293 survey in Kainji Lake National Park (Nigeria), the second site visited, all samples revealed SA
294 concentrations around or below the detection threshold (grey background). This is remarkable
295 because the substrate controls from different days and locations showed an average SA
296 concentration of 12 µM (dotted line), and this concentration of free SA present in the substrates
297 could not be recovered in any of the specimen collected at the same sites. This finding let us to
298 incorporate a free SA control in all subsequent field screenings to ensure that hydrolysed SA would
299 be stable in the homogenized tissue sample and not be enzymatically or chemically degraded.

300 **3.2. Liquid sialidase assay**

301 We assayed whole bacteria cultures, culture supernatants and bacteria lysates for sialidase activity.
302 In several samples, addition of $CaCl_2$ seemed to enhance sialidase activity in the culture
303 supernatant samples (Fig. S3). Analysis of Neu5Ac-MU cleavage revealed potential sialidase
304 activity in several bacteria isolates.



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306 **Figure S3. Neu5Ac-MU cleavage by bacterial isolates.** Bacterial culture supernatants (A, upper), whole
307 bacterial cultures (B, middle) and bacteria lysates (C, lower) were incubated with 1 mM Neu5Ac-MU with
308 and without 4 mM $CaCl_2$ and fluorescence intensity was measured after 18 hours of incubation. Bacteria

culture supernatants were applied either undiluted or 1:2 diluted. Control samples were simultaneously incubated with 10 µM MU instead of Neu5Ac-MU to determine any interference of the sample with fluorescence detection. The fluorescence values were corrected for the deviation of the MU fluorescence intensity and for background fluorescence of the substrates resulting from presence of free MU within Neu5Ac-MU.

3.3. Cloning of the *Paenibacillus sp.* sialidase gene

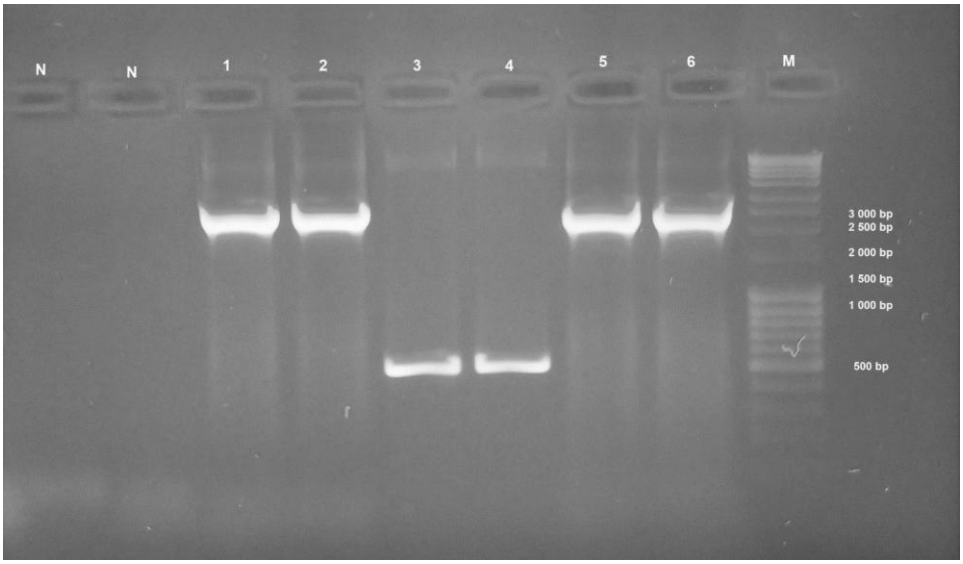

Figure S4. Colony PCR amplicons of different portion of the construct. The lane (M) shows the marker. The lanes (N) show the negative control. The lanes 1 and 2 show the entire gene, amplified from the promoter to the terminator (3 008 bp). The lanes 3 and 4 show a portion of the gene (541 bp). The lanes 5 and 6 show the amplification from a portion of the gene to the terminator (2732 bp)

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