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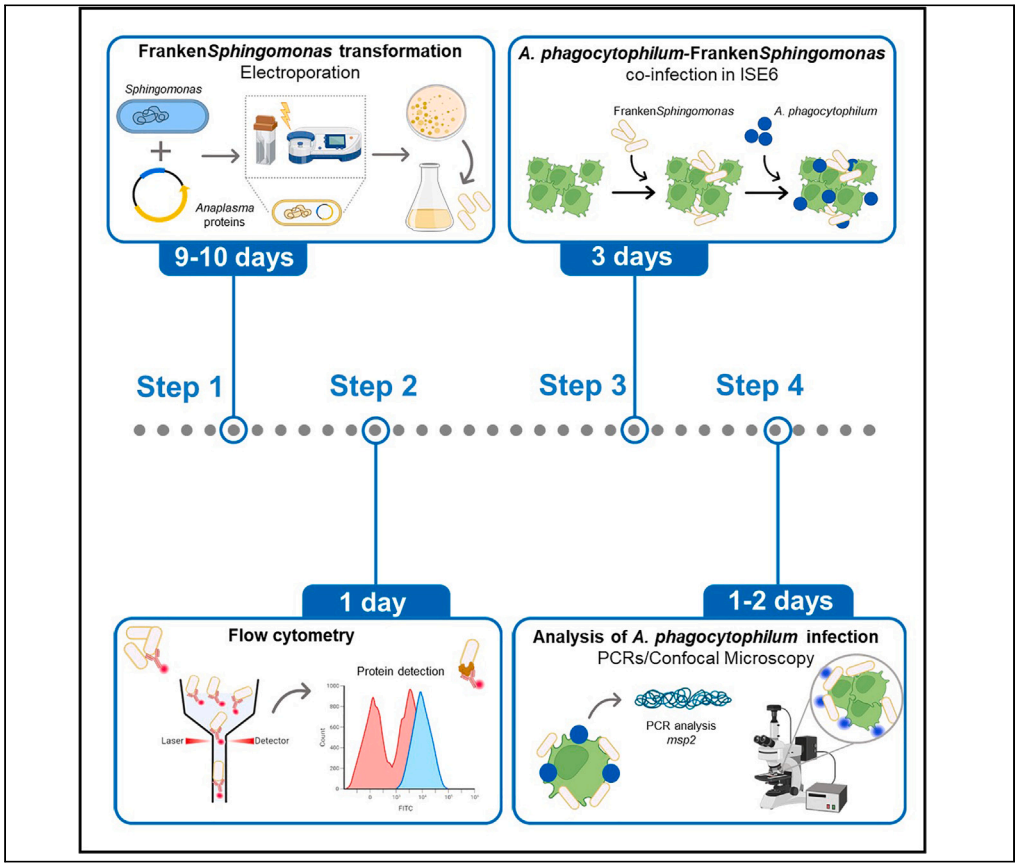
Genetic modification, characterization, and co-infection of Franken *Sphingomonas* and *Anaplasma phagocytophilum* in tick cells



Paratransgenesis through genetic manipulation of symbiotic or commensal microorganisms has been proposed as an effective and environmentally sound approach for the control of vector-borne diseases, including tick bite-related pathologies, and reducing pathogen transmission. Here, we present a protocol for *Sphingomonas* transformation with *Anaplasma phagocytophilum* major surface protein 4 and heat shock protein 70. We describe a step-by-step protocol for *in vitro* study of interactions between transformed Franken *Sphingomonas* and *Ixodes scapularis* ISE6 tick cells during *A. phagocytophilum* infection.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Frankenbacteriosis is an effective approach for the control of vector-borne diseases

Paratransgenic modification of commensal bacteria to mimic pathogen

Production of Franken *Sphingomonas* with *A. phagocytophilum* MSP4 and HSP70 proteins

Control of pathogen infection in tick cells using Franken *Sphingomonas*

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Genetic modification, characterization, and co-infection of Franken *Sphingomonas* and *Anaplasma phagocytophilum* in tick cells

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SUMMARY

Paratransgenesis through genetic manipulation of symbiotic or commensal microorganisms has been proposed as an effective and environmentally sound approach for the control of vector-borne diseases, including tick bite-related pathologies, and reducing pathogen transmission. Here, we present a protocol for *Sphingomonas* transformation with *Anaplasma phagocytophilum* major surface protein 4 and heat shock protein 70. We describe a step-by-step protocol for *in vitro* study of interactions between transformed Franken *Sphingomonas* and *Ixodes scapularis* ISE6 tick cells during *A. phagocytophilum* infection.

For complete details on the use and execution of this protocol, please refer to Mazuecos et al. (2023).[1]

BEFORE YOU BEGIN

This protocol provides detailed instructions for genetic modification of symbiotic bacteria *Sphingomonas* spp. with proteins of the pathogenic bacteria *Anaplasma phagocytophilum* that will be expressed on *Sphingomonas* bacteria surface (herein, Franken *Sphingomonas*). Moreover, it included details for characterization of interactions between *Ixodes scapularis* ISE6 tick cells and Franken *Sphingomonas* during pathogen infection. Flow cytometry and confocal microscopy were used as molecular tools for validation of bacterial transformation and co-infection. The *A. phagocytophilum* major surface protein 4 (MSP4) and heat-shock protein 70 (HSP70), both proteins selected for paratransgenesis, have been shown to be involved in tick-pathogen and host-pathogen functional interactions.[2,3] The protocol below describes the specific steps and cell culture conditions for investigating interactions in ISE6 tick cells during co-infection of Franken *Sphingomonas* and *A. phagocytophilum*. We have also used this protocol for co-infection in human HL-60 cell line and can be extrapolated to other pathogen infections under specific-pathogen infection conditions.

Sphingomonas SpAR92 strain: Growth and bacterial culture conditions

Timing: 1–2 days

1. Grow *Sphingomonas* sp. AR92 (SpAR92) strain at 30°C in 250 mL of sterilized broth R2A medium for 24–48 h with shaking (200 rpm) until medium color turn a strong yellow. The bacterial strain can be obtained from Colección Española de Cultivos Tipo (Valencia, Spain) (https://www.uv.es/uvweb/coleccion-espanola-cultivos-tipo/es/coleccion-espanola-cultivos-tipo-1285872233521.html).

R2A medium		
Reagent	Final concentration (g/L)	Amount
Yeast extract	0.5	0.5 g
Proteose Peptone	0.5	0.5 g
Casein Hydrolase	0.5	0.5 g
Glucose	0.5	0.5 g
Soluble Starch	0.5	0.5 g
Sodium Pyruvate ($C_3H_3NaO_3$)	0.3	0.3 g
Dipotassium phosphate (K_2HPO_4)	0.3	0.3 g
Magnesium sulfate ($MgSO_4$)	0.05	0.05 g
ddH_2O	N/A	Adjust to 1 L
Total	N/A	1 L

Note: Sterilize the solution by autoclaving at 121°C for 15 min and store at 4°C in the dark. R2A medium could be stored at 4°C for 2–3 months, but it is highly recommended to use fresh media (1–2 weeks stored maximum) for growing *Sphingomonas*.

Note: It is highly recommended to regularly measure *Sphingomonas* bacterial growth by absorbance (O.D. 600 nm) every 8–10 h during bacterial culture to set an optimal bacterial growth time under specific laboratory conditions.

Optional: *Sphingomonas* appearance is yellow (see examples in https://www.flickr.com/photos/nathanreading/6941968519 and https://www.mdpi.com/2073-4395/10/11/1673). If a strong yellow color of medium during bacterial growth is not observed after 48 h or the medium appears white and cloudy with bubbles, it is recommended to grow bacteria on R2A agar plates for 24 h at 30°C first, and then, inoculate several colonies in R2A broth medium as described above.

Ixodes scapularis ISE6 tick cell culture

Timing: 4–5 days

The ISE6 embryonic tick cells are a stabilized cell line from *Ixodes scapularis*. The ISE6 cultures were maintained according to previously described methods[4–6] in the appropriate tick cell culture medium L-15B300, as reported by Munderloh et al.[7] and as follows.

2. Preparation of L-15B300 medium for cell culture.
   a. Prepare a trace mineral stock solution (solutions A, B and C) and use them to prepare the stock solution D:

Trace mineral Stock Solution		
Reagent	Amount	Final Molarity in L-15B
Stock solution A		
$CoCl_2 \cdot 6H_2O$	20 mg	8.4×10^{-9}

(*Continued on next page*)

Continued		
Reagent	Amount	Final Molarity in L-15B
$CuSO_4 \cdot 5H_2O$	20 mg	8×10^{-9}
$MnSO_4 \cdot H_2O$	160 mg	9.5×10^{-8}
$ZnSO_4 \cdot 7H_2O$	200 mg	7×10^{-8}
Distilled water	Up to 100 mL	N/A
Total	100 mL	
Stock Solution B		
Na_2MoO_4	20 mg	8.3×10^{-9}
Distilled water	Up to 100 mL	N/A
Total	100 mL	
Stock Solution C		
Na_2SeO_3	20 mg	1.2×10^{-8}
Distilled water	Up to 100 mL	N/A
Total	100 mL	
Stock Solution D		
Glutathione	1000 mg	3.3×10^{-5}
Ascorbic acid	1000 mg	7.5×10^{-5}
$FeSO_4 \cdot 7H_2O$	50 mg	1.8×10^{-6}
Stock Solution A	1 mL	N/A
Stock Solution B	1 mL	N/A
Stock Solution C	1 mL	N/A
Distilled water	Up to 100 mL	N/A
Total	100 mL	

Note: Reagents for the stock solutions should be dissolved in distilled water in the order listed, and the volume of each stock solution should be brought up to 100 mL. Store aliquots of stock solution D and the vitamin stock in 1 mL aliquots at −20°C. Stock solutions A, B and C can be stored directly at −20°C.

Important: The medium should not be autoclaved. Once completed medium has been prepared, it should be sterilized by filtration.

b. Prepare the vitamin stock solution:

Vitamin Stock Solution		
Reagent	Amount	Final Molarity in L-15B
p-aminobenzoic acid	100 mg	7.3×10^{-3}
Cyanocobalamine (B_{12})	50 mg	3.7×10^{-4}
d-Biotin	10 mg	4.1×10^{-4}
Total	100 mL	

Note: Vitamin Stock solution can be stored at −20°C.

c. Prepare L-15B medium: Dissolve Leibovitz's L-15 medium, powder for 1 L medium (#41300-021, Invitrogen) in 900 mL distilled water. Then add the following components:

L-15B medium		
Reagent	Amount	Final Molarity in L-15B
Aspartic acid	299 mg	2.25×10^{-3}
Glutamic acid	500 mg	3.40×10^{-3}
Proline	300 mg	2×10^{-3}
α-ketoglutaric acid	299 mg	2.05×10^{-3}

(*Continued on next page*)

Continued		
Reagent	Amount	Final Molarity in L-15B
D-glucose	2239 mg	12.43×10^{-3}
Trace Mineral Stock Solution D	1 mL	N/A
Vitamin Stock Solution	1 mL	N/A
Distilled water	~900 mL	
Total	1000 mL	

Note: Store at 4°C up to 4 months. For long-term storage, keep at −20°C.

d. Add half of the volume of water specified in the table above and mix without warming at 200 rpm in a stirrer or for 15–20 min until reagents are completely dissolved.
e. Bring the volume up to 1 L and sterilize by filtration (0.22 μm). f. Prepare L-15B300 medium with the following reagents:

L-15B300 medium	
Reagent	Volume
L-15B medium	65 mL
Tryptose phosphate broth	10 mL
Fetal calf serum	5 mL
10% lipoprotein-cholesterol solution concentrate (#ICN19147625, MP Biomedicals)	1 mL
Glutamax	1 mL
Milli-Q water	20 mL
Total	102 mL

Note: Prepare a 10% lipoprotein-cholesterol solution stock by taking 10 mL of Lipoprotein-cholesterol concentrate and add 90 mL of L-15B medium. Filter with a 0.22 mm filter, make aliquots and store at −20°C

Note: Adjust pH to a light orange color using sterile 1M NaOH. Based on our experience, orange color corresponds to a pH between 6.5 and 6.9. Before adjusting the pH, we obtain a pH between 4.3 and 4.5.

3. Culture of ISE6 tick cells.
   a. Incubate ISE6 tick cells in an atmosphere of ordinary air in flat-side tubes (Nunc, Cat# 156758) and dry incubators at 31°C.
   b. Change cell culture medium (L-15B300, Step 2) once a week by removal and replacement of 50%–80% of culture medium volume.
   c. Since ISE6 tick cells are adherent, resuspend by pipetting (Figure 1).

⚠ CRITICAL: When culture medium is changed, the volume removed is added to a new flask for overnight pre-conditioning (pre-conditionate flask). The parent flask (the flask with subculture is going to be performed) is always maintained and can be used for future subcultures over several months or even years.

Note: We recommend to check cells under the microscope before each change to discard potential contaminations and estimate cells confluence.

⚠ CRITICAL: Trypsin or EDTA is not recommended at early passages. Scraping or washing with a flow of medium is also an optional strategy for passaging cells.

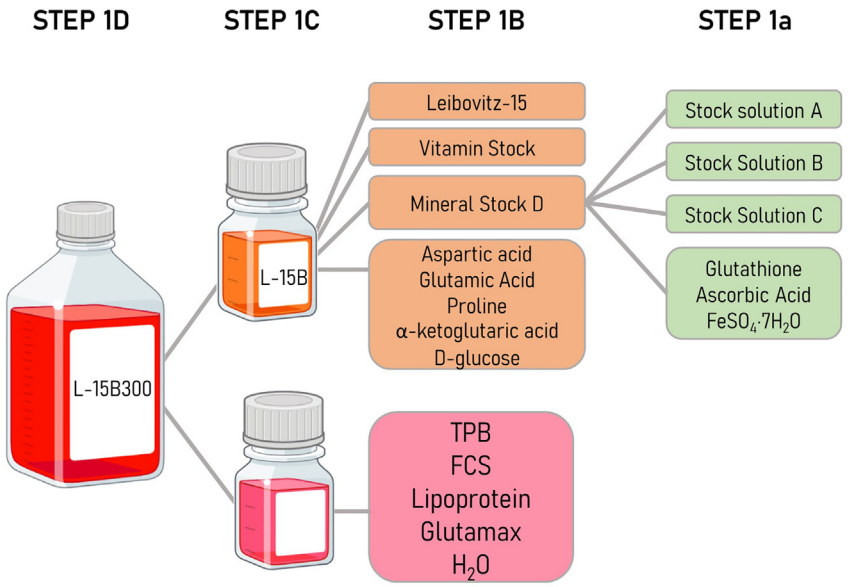

Figure 1. Representative scheme of L-15B300 preparation

4. Subcultures of ISE6 tick cells.
   a. The appropriate cell suspension volume is transferred to a new pre-conditionate flask. Based on our experience, with transfer 1.5 mL to a new pre-conditionate flask from a 3 mL culture tube (1:2 dilution).

   Note: Subcultures must be done every 3–4 weeks when ISE6 tick cells are confluent.

Semi-purified *A. phagocytophilum* isolation

Timing: 3–5 days

For *A. phagocytophilum* isolation and consequently co-infection with Franken *Sphingomonas*, we describe here a protocol for *A. phagocytophilum* isolation starting from pathogen-infected human HL-60 culture cells.

5. Inoculate ISE6 tick cells with *A. phagocytophilum* (NY18 human isolate)-infected HL-60 cells.

   Note: Infected HL-60 cells have been cultured in our laboratory for more than 10 years as described.[8] Details of culture conditions are available from the lead contact upon request.

   Optional: *A. phagocytophilum* can be isolated directly and infected to our target cells from HL-60-infected culture. However, it is recommended to previously infect *A. phagocytophilum* to the cell culture of our target (ISE6 cell line) for pathogen adaptation to culture conditions.

6. Collect *A. phagocytophilum*-infected ISE6 tick cells at 70%–80% infection.

   Note: Infection can be monitored daily by Giemsa staining[3] in which intracellular morulae (intracellular inclusions) are detected in smears after cytospin or centrifugation (Figures 2 and 3) or by qPCR targeting *A. phagocytophilum* major surface protein *msp2* or *msp4* genes

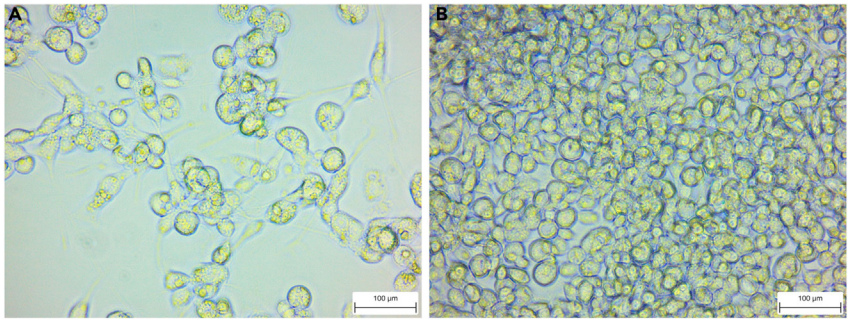

Figure 2. Detection of *A. phagocytophilum*-infected ISE6 tick cells

(A and B) Giemsa staining for detection of intracellular morulae (intracellular inclusions) in (A) ISE6 tick non-confluent attached and (B) confluent cells.

followed by DNA isolation by DNeasy Blood & Tissue kit following manufacturer's instructions (#69504, QIAGEN).

Note: Under our laboratory routine, Giemsa staining is weekly done to observe bacterial infection in our cell lines (human and tick cell lines). A qPCR targeting *A. phagocytophilum* proteins as *msp2* or *msp4* genes is done previous an experiment with these cell culture lines is performed to validate infection with this sensitive and robust technique and proportionate data of infection levels.

7. For isolation of cell-free bacteria from cell culture, pass *A. phagocytophilum*-infected ISE6 tick cells 5–10 times through a 21-gauge syringe to disrupt cell membranes.
8. Centrifugate at 300 × *g* for 5 min and collect supernatant.
9. Quantify bacteria by absorbance at O.D. 600 nm using a spectrophotometer or microplate reader with appropriate media as blank

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-MSP4 primary antibody (1:50)	This paper	N/A
Monoclonal anti-HSP70 primary antibody (1:100)	Sigma-Aldrich	Cat# H5147
Anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (1:100)	Sigma-Aldrich	Cat# RABHRP1
Anti-rabbit IgG FITC-conjugated antibody (1:100)	Sigma-Aldrich	Cat# AP106F
Fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (1:100)	Abcam	Cat# ab6717
Goat anti-rabbit IgG FITC (1:100)	Sigma-Aldrich	Cat# F0382
Goat-anti mouse IgG FITC (1:100)	Sigma-Aldrich	Cat# F2012
Bacterial and virus strains		
A. phagocytophilum NY18	NY18 human isolate	N/A
Sphingomonas sp. AR92 (SpAR92)	Colección Española de Cultivos Tipo (Paterna, Valencia, Spain)	CECT 7178
Chemicals, peptides, and recombinant proteins		
Gibco Leibovitz L-15 medium	Thermo Fisher Scientific	Cat# 41300021
$CoCl_2 \cdot 6H_2O$	Sigma-Aldrich	Cat# C8661
$CuSO_4 \cdot 5H_2O$	Sigma-Aldrich	Cat# C3036
$MnSO_4 \cdot H_2O$	Sigma-Aldrich	Cat# M7899

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
$ZnSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# Z0251
Na_2MoO_4	Sigma-Aldrich	Cat# M1651
Na_2SeO_3	Sigma-Aldrich	Cat# S5261
Glutathione	Sigma-Aldrich	Cat# G6013
Ascorbic acid	Sigma-Aldrich	Cat# A4544
$FeSO_4 \cdot 7H_2O$	Sigma-Aldrich	Cat# F8633
p-aminobenzoic acid	Sigma-Aldrich	Cat# 205-753-0
Cyanocobalamine	Sigma-Aldrich	Cat# 47869
D-biotin	Sigma-Aldrich	Cat# 2031
D-aspartic acid	Sigma-Aldrich	Cat# 219096
Glutamic acid	Sigma-Aldrich	Cat# 49449
Proline	Sigma-Aldrich	Cat# 81709
α-ketoglutaric acid	Sigma-Aldrich	Cat# K1750
D-glucose	Sigma-Aldrich	Cat# G7021
Tryptose phosphate broth	Gibco	Cat# 18050-039
Fetal calf serum	Gibco	Cat# 10500-064
Bovine lipoprotein (ICN)	MP Biomedicals	Cat# 191476
Glutamax	Gibco	Cat# 35050-038
LB broth	Avantor VWR	Cat# J106
LB agar	Avantor VWR	Cat# 84684.0500
Tetracycline	Sigma-Aldrich	Cat# 87128
Kanamycin	Sigma-Aldrich	Cat# K1377
Agar	Sigma-Aldrich	Cat# A1296
R2A broth	HiMedia	Cat# M1687
R2A agar	Merck	Cat# 1.00416
0.2 cm cuvettes	Bio-Rad	Cat# 1652086
TMB (3,3′,5,5′-tetramethylbenzidine)	Promega	Cat# W4121
ProLong Antifade reagent containing DAPI	Thermo Fisher Scientific	Cat# P36962
Streptomycin-Penicillin	Thermo Fisher Scientific	Cat# 15140122
Vanillate (4-hydroxy-3-methoxybenzoic acid)	Sigma-Aldrich	Cat# 659282
SOC medium	Invitrogen	Cat# 15544034
Triton X-100	Sigma-Aldrich	Cat# X100RS
Tween 20	Sigma-Aldrich	Cat# P9416
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A9418
ProLong Diamond Antifade Mountant	Thermo Fisher Scientific	Cat# P36961
Invitrogen DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride)	Thermo Fisher Scientific	Cat# 10184322
PBS 10 mM	Sigma-Aldrich	Cat# P3813
Critical commercial assays		
Promega PCR master mix	Promega	Cat# M7505
MiniElute PCR Purification	QIAGEN	Cat# 28004
GeneJet Plasmid Kit	Thermo Fisher Scientific	Cat# K0502
pENTR/D-TOPO Cloning kit	Invitrogen	Cat# K240020
Gateway LR Clonase II kit	Thermo Fisher Scientific	Cat# 11791020
Intracell Solution kit	Immunostep	Cat# INTRA-V500T
DNeasy Blood & Tissue kit	QIAGEN	Cat# 69504
Luna Universal qPCR Master Mix	New England Biolabs	Cat# M3003
Experimental models: Cell lines		
Ixodes scapularis ISE6 tick cell line	Provided by U. G. Munderloh (University of Minnesota) and L. Bell-Sakyi (Tick Cell Biobank, University of Liverpool)	N/A
Recombinant DNA		
Plasmid: pVHD vector	Addgene (from Julia Vorholt)	#61303 http://n2t.net/addgene:61303; RRID: Addgene_61303

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software		
GraphPad Prism Software	Dotmatics	RRID: SCR_002798 https://www.graphpad.com/
FlowJo Software	Becton Dickinson	RRID: SCR_008520 https://www.flowjo.com/solutions/flowjo/downloads
Other		
Thermocycler	N/A The one available in the work laboratory	N/A
Spectrophotometer	N/A The one available in the work laboratory	N/A
Incubator with agitation	N/A The one available in the work laboratory	N/A
Thermomixer	N/A The one available in the work laboratory	N/A
NanoDrop	N/A The one available in the work laboratory	N/A

MATERIALS AND EQUIPMENT

Agar Plates		
Agar plate	Antibiotic	Final concentration
LB agar	Kanamycin	50 μg/mL
LB agar	Tetracycline	20 μg/mL
R2A agar	Tetracycline	20 μg/mL

Other solutions	
Name	Reagents, preparations and storage
250 μM Vanillate	Prepare a 250 mM stock by adding 2.1 g of Vanillate to 50 mL of ethanol. Store at −20°C.
4% paraformaldehyde (PFA)	Add 40 g of paraformaldehyde to 800 mL PBS. Stir the mixture at 60°C in ventilation hood. To completely dissolve PFA, add 1M NaOH drop by drop until a clear solution is formed and adjust pH to 6.9–7.4. Allow mix to cool down at room temperature. 4% paraformaldehyde can be stored at 4°C for 1 week. It is recommended to prepare fresh solution the day of the experiment.
0.25% Triton X-100	Add 25 μL Triton X-100 to 10 mL PBS 1×. Store at 4°C
PBS-0.1% Tween 20 (PBS-T20)	Add 100 μL Tween 20–100 mL PBS 1×. Store at 4°C.
1% BSA-PBS-0.1% Tween 20 (1% BSA-PBS-T20)	Add 1 g BSA to 100 mL PBS-0.1% Tween 20.

STEP-BY-STEP METHOD DETAILS

Generation of Franken *Sphingomonas* by genetic modification of *Sphingomonas* SpAR92

Timing: 9–10 days

In this section, genetic modification of *Sphingomonas* SpAR92 with two *A. phagocytophilum* proteins, MSP4 and HSP70, is detailed. Three molecular and consecutive transformations are required to obtain Franken *Sphingomonas*-X (X = target protein) namely, Franken *Sphingomonas*-MSP4 or Franken *Sphingomonas*-HSP70. A representative scheme of the following steps is disclosed at the end of this section (Figures 4 and 5).

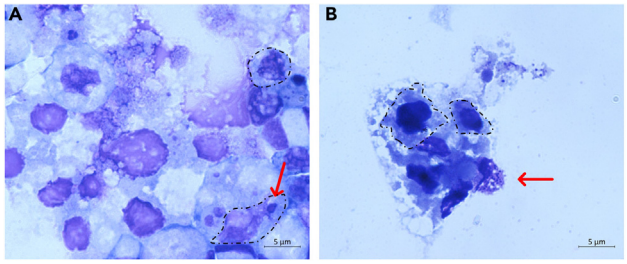

Figure 3. Giemsa staining of *A. phagocytophilum*-infected ISE6 tick cells

(A and B) Red arrows represent (A) initial formation of *A. phagocytophilum* colonies and (B) the rupture of colonies releasing corpuscles from ISE6 tick cells. Black dotted line represents an example of a delineated cell.

1. To generate PCR products for transformation, amplify *A. phagocytophilum* (strain NY18) *hsp70* (KX891324.1) and *msp4* (KP861635.1) genes from DNA extracted from *A. phagocytophilum* inoculum by PCR using the following specific primers and annealing conditions.

Primer Sequences for *msp4* and *hsp70* gene amplification

Gene	Primer pairs (F, forward; R, reverse)	Annealing conditions	Amplicon size
msp4	F: 5′- CACCATGAATTACAGAGAATTGCTTGTA-3′ R:5′-CTAATTGAAAGCAAATCTTGCTCCTAT-3′	52°C 60 s	846 bp
hsp70	F: 5′-CACCATGGCGGCTGAGCGTATAATAGGT-3′ R: 5′-CTAAGTATTCTTCTTGTCCTCGGCCTT-3′	52°C 60 s	1935 bp

PCR reaction master mix

Reagent	Amount
PCR Master Mix 2×	25 μL
DNA template (3–5 μL)	20 ng
Primer Forward (10 μM)	1 μL
Primer Reverse (10 μM)	1 μL
ddH_2O	Up to 50 μL
Final volume	50 μL

Note: PCR reaction mix from PCR Master Mix, Promega (https://www.promega.es/products/pcr/taq-polymerase/master-mix-pcr/?catNum=M7505#protocols).

a. Run an agarose gel (1.25%) with 4 μL of PCR products to validate amplification and amplicon size (100 mA, 30–45 min).
b. Purify PCR products following manufacturer protocols using MiniElute PCR purification Kit (QIAGEN).

⚠ CRITICAL: To elute DNA during MiniElute PCR purification kit protocol, elute up to 10 μL Buffer EB (10 mM Tris-Cl, pH 8.5) to ensure higher DNA concentration for cloning. A further dilution might be done if concentration is too high for volume processing.

c. Quantify DNA concentration using a NanoDrop and dilute to an optimal working concentration (if needed). We recommend to dilute DNA solution to a 5–10 ng/μL.

Pause point: PCR product can be stored at −20°C until further processing.

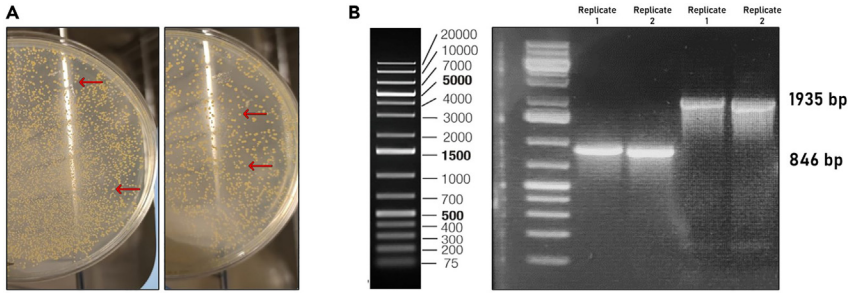

Figure 4. Genetic modification of *Sphingomonas* SpAR92 with two *A. phagocytophilum* proteins, MSP4 and HSP70

(A) Representative SpAR92 bacterial colonies transformed by electroporation (red arrows point to single colonies transformed by electroporation on a R2A agar plates with tetracycline).

(B) Representative 1.25% agarose gel of two PCR products of *msp4* (846 bp) and *hsp70* (1935 bp) genes isolated from SpAR92 colonies for validation of positive colonies.

2. Clone resulting purified PCR products (5–10 ng of fresh PCR product in a final volume of 0.5–4 μL) using a pENTR Directional TOPO cloning kit (Thermo Fisher Scientific) in *Escherichia coli* TOP10 competent cells following manufacturer protocols Thermo Fisher (https://www.thermofisher.com/order/catalog/product/K240020). pENTR/D-TOPO plasmid includes a cassette for kanamycin resistance.
   a. Perform TOPO cloning reaction by chemical transformation.

Note: Manufacturer's instructions recommend for optimal results a 0.5:1 to 2:1 molar ratio of PCR product:TOPO vector. 5–10 ng of fresh PCR product in a final volume of 2–4 μL should be enough to succeed in chemical transformation.

TOPO cloning reaction.

Reagent	Amount
Fresh PCR product	2–4 μL (5–10 ng)
Salt solution	1 μL
TOPO vector	1 μL
Sterile water	Up to 6 μL
Final Volume	6 μL

   b. Mix the TOPO reaction gently and incubate on ice for 15–20 min.
   c. Add 2 μL of the TOPO cloning reaction to 1 vial of One Shot Chemically Competent *E. coli* cells and mix gently by vortex for 15 s.
   d. Incubate on ice for 20 min.
   e. Heat-shock on a thermoblock for 30 s at 42°C without shaking. Immediately, transfer the mix to ice.
   f. Add 250 μL of SOC medium prior equilibrated to room temperature (18–25°C).

Optional: If SOC medium is not available, fresh LB broth equilibrated to room temperature without antibiotics is also optimal in this step.

   g. Incubate the transformed cells-containing tube horizontally at 37°C for 1.5 h with gentle shaking (200 rpm).
   h. Spread cells on LB agar plates enriched with kanamycin at 50 μg/mL.

Note: It is recommended to spread plates with different volumes (50 μL and 100 μL) of cells mix to optimize transformation and obtained colonies.

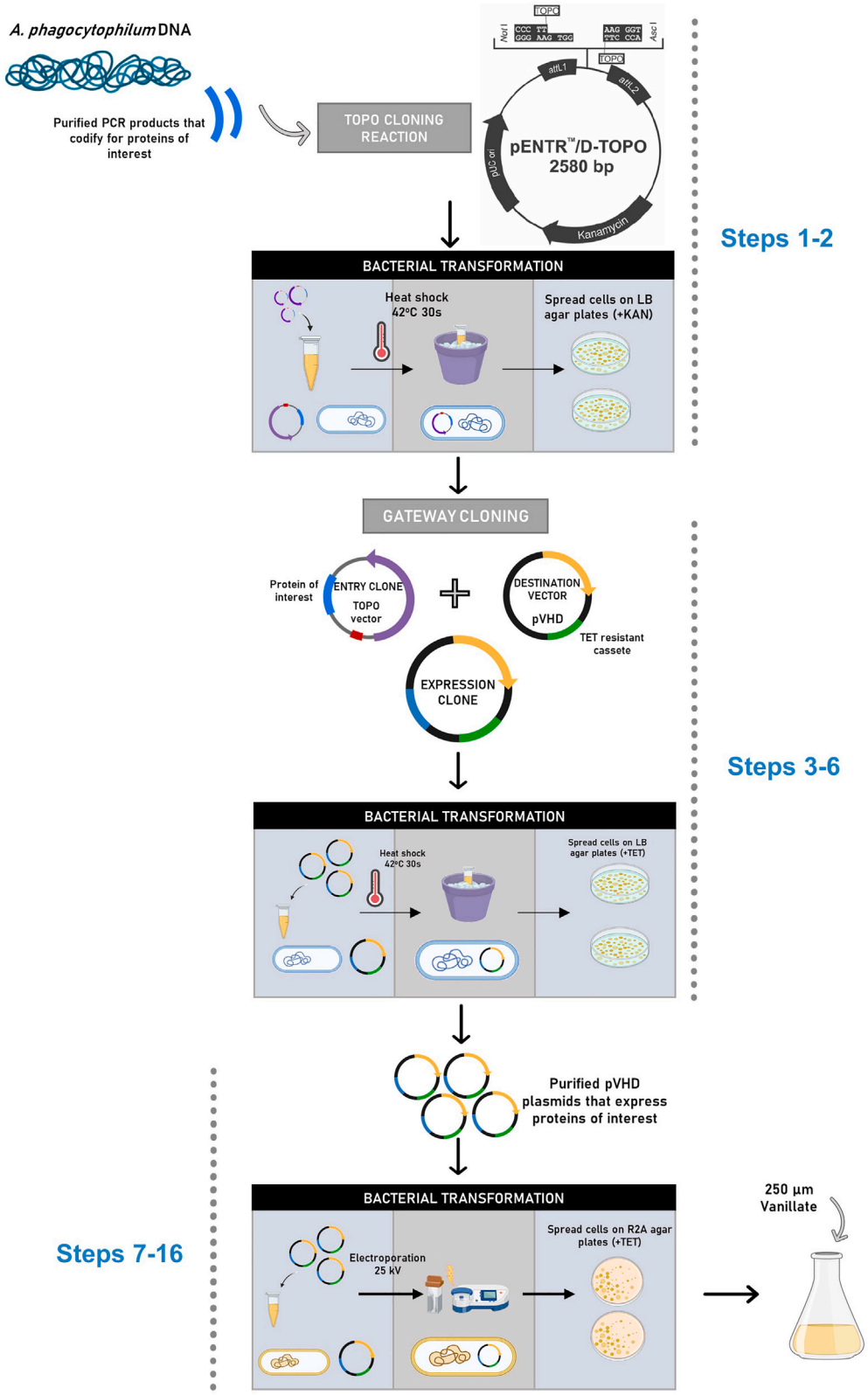

Figure 5. Representative workflow for Steps 1-16

i. Incubate plates overnight at 37°C.
j. On the following day, pick 5–6 antibiotic-resistant colonies and plate again on new LB agar plates with kanamycin at 50 μg/mL.
k. Analyze PCR fragment insertion of positive colonies by PCR as described above (Step 1) and validate by running a 1.25% agarose gel.
l. Grow positive colonies in a small volume (10 mL) of LB broth with kanamycin (50 μg/mL) overnight in replicates at 37°C.

Note: Grow colonies in at least two replicates. One intended for bacterial stock stored at −80°C, and the other one for plasmid purification using GeneJet Plasmid kit following manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Flr_clonaseii_man.pdf)

m. Centrifuge bacterial cultures at 2000 × *g* for 15 min at 4°C.
n. Purify the plasmid of interest using GeneJet Plasmid kit, quantify DNA concentration and store at −20°C until further processing.

3. Generate an expression construct using plasmid pVHD (Addgene plasmid #61303) herein, pVHD, pVHD-msp4 and pVHD-hsp70) by performing LR recombination reaction (recombination reaction between attL and attR sites) between the entry clone and a Gateway destination vector.
   a. For *Sphingomonas* transformation, a vanillate-inducible gene expression is chosen as destination vector for Gateway cloning (tetracycline resistance plasmid). Plasmid details are available: https://www.addgene.org/61303/
   b. Perform Gateway Cloning following manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assehttps://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Flr_clonaseii_man.pdfts.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Flr_clonaseii_man.pdf).

Note: For the entry clone (TOPO vector), it is recommended to add 100 ng in a final volume of 5 μL to the LR reaction to obtain an appropriate and working number of colonies.

4. Transform One Shot Chemically Competent *E. coli* cells as described above (Step 2) with 1 μL of each LR reaction.
5. Spread cells on LB agar plates enriched with tetracycline 20 μg/mL and incubate overnight at 37°C.
6. Pick 5–6 antibiotic-resistant colonies and validate gene insertion by PCR. Grow positive colonies in a small volume (10 mL) of LB broth with tetracycline at 20 μg/mL in replicates overnight (troubleshooting: problem 1).
7. Purify the plasmid of interest (pVHD-msp4 or pVHD-hsp70) using GeneJet Plasmid kit, quantify its concentration by NanoDrop (or microplate reader) and store at −20°C until further processing.

Note: To ensure an accurate expression construct, it is highly recommended to perform a restriction analysis and sequence DNA from positive transformed *E. coli* colonies once the plasmid of interest is purified.

8. Grow *Sphingomonas* SpAR92 at 30°C in 250 mL of sterilized broth R2A medium until O.D. 600 nm reaches 0.7–0.8.
9. Centrifuge 50 mL of the bacterial culture at 2000 × *g* for 15 min at 4°C.
10. Wash pellet twice with 10 mL of chilled 10% glycerol.
11. Resuspend pellet in a small volume (400–500 μL) in chilled 10% glycerol.
12. Mix 100 μL of the SpAR92 cell suspension with 1 μg of the purified expression construct.

Note: It is recommended to add 1 μg of purified expression construct in 3–4 μL to the cell suspension. Then, after quantify plasmid purified concentration on Step 7, prepare a DNA dilution around 0.2–0.4 μg/μL.

13. Introduce purified expression construct generated into *Sphingomonas* strain by electroporation[9] to obtain Franken *Sphingomonas:*
    a. Put bacterial-expression construct mix (Step 12: 100 μL of SpAR92 cell suspension and 1 μg of purified expression constructs) into 0.2 cm electroporation cuvettes.
    b. Perform electroporation using a Gene Pulser (or similar) with a single pulse at 2.5 kV.
    c. Immediately, carefully add 600 μL of SOC medium at room temperature for recovering directly to the cuvette.
    d. Transfer the cell suspension to a new 2-mL tube and allow cells to grow horizontally for 2 h at 30°C with gentle shaking (200 rpm) (troubleshooting: problem 2).
    e. Spread cells on R2A agar plates with tetracycline(Step 3, resistance cassette included in p-VHD plasmid) at 20 μg/mL for colony selection.
    f. Incubate plates overnight at 30°C.

Note: Depending on the purpose of the experiment, it is recommended to additionally transform *Sphingomonas* with pVHD destination vector with no insert fragment (pVHD plasmid alone) to have a good experimental control in the experiment (herein, *Sphingomonas*-pVHD).

14. On the following day, pick 5–6 antibiotic-resistant colonies and plate again on new R2A agar plates with tetracycline at 20 μg/mL.
15. Validate positive colonies carrying pVHD, pVHD-msp4 and pVHD-hsp70 (Franken *Sphingomonas*-pVHD, Franken *Sphingomonas*-msp4, Franken *Sphingomonas*-hsp70 plasmids by PCR as described (Step 1) and 1.25% agarose gel.
16. Grow SpAR92 positive colonies in R2A broth with tetracycline at 20 μg/mL at 30°C to mid-exponential phase (O.D. 600 nm = 0.6–0.8) in a small volume (50 mL) and induce gene expression by the addition of 250 μM Vanillate for at least 6 h.

Note: Grow SpAR92 positive colonies in at least two replicates. One intended for preparing bacterial stocks to be further stored at −80°C and the other one for characterization of Franken *Sphingomonas*.

Note: If characterization and co-infection assay are going to be done continuously, grow SpAR92 colonies in at least three replicates of two different volumes: 50 mL for bacterial stock, 50 mL for characterization by flow cytometry and 250 mL for co-infection assay.

Pause point: If the researcher does not have time to continue with the following steps continuously, a bacterial stock at −80°C can be defrosted, inoculated in 250 mL of R2A broth with antibiotic (tetracycline 20 μg/mL) and grow at 30°C with shaking to mid-exponential phase (O.D. 600 nm = 0.6–0.8) for further characterization.

⚠ CRITICAL: the time for inducing gene expression depends on target protein, bacterial culture and plasmid introduced in the cell suspension. It is recommended to set an appropriate induction time for each protein. Below, is described a protocol for setting gene expression induction time in Franken *Sphingomonas* by flow cytometry.

Characterization of Franken *Sphingomonas* by flow cytometry

Timing: 1–2 days

In this section, a detailed protocol for characterization of Franken *Sphingomonas* by flow cytometry to analyze targeted protein production (herein, MSP4 and HSP70 proteins) in permeabilized cells at different induction times (0, 3 and 6 h) is described. Target protein could be located on the surface or inside cells.

17. Induce gene expression by addition of 250 μM Vanillate to 100 mL of Franken Sphingomonas-pVHD, Franken *Sphingomonas*-MSP4 and Franken *Sphingomonas*-HSP70 bacterial cultures at O.D. 600 nm = 0.6–0.8.
18. Take 1-mL aliquots in replicates (3–4 replicates for each bacterial culture and induction time) at 0, 3 and 6 h induction times.

Note: If needed, dilute aliquots until an O.D. 600 nm = 0.6 is reached with PBS +1% FBS.

19. Wash bacterial cultures twice with PBS +1% FBS by centrifugation for 5 min at 7000 × *g* at room temperature.

Note: For Franken *Sphingomonas* transformed with pVHD plasmid alone, directly resuspend pellet as described in Step 3. This experimental condition will be used as a control for flow cytometry with no-protein detection.

20. For non-permeabilized replicates:
    a. Incubate each separate cell suspension (Franken *Sphingomonas*-MSP4 and Franken *Sphingomonas*-HSP70) with 50 μL of rabbit anti-MSP4 primary antibody (1:50, 0.4 mg/mL) and monoclonal anti-HSP70 primary antibody (1:100, 0.7 mg/mL), respectively for each bacterial culture in PBS +5% FBS for 2 h at room temperature.
    b. Wash by adding 1 mL PBS +1% FBS and centrifugate for 5 min at 7000 × *g*.
    c. Incubate with respective secondary antibody (goat anti-rabbit IgG FITC for MSP4 and goat anti-mouse IgG FITC for HSP70) at 1:100 dilution for 1 h at room temperature in the dark.

⚠ CRITICAL: Do not forget to add Franken *Sphingomonas* with no antibody (Franken*Sph*-MSP4 and Franken*Sph*-HSP70) and with only respective secondary antibodies as background control for cytometry analysis for non-permeabilized cells.

21. For permeabilized cells, use Intracell Solution kit (https://shop.immunostep.com/accesory-reagents/1281-intracell.html) following manufacturer's instructions with some modifications:
    a. Add 100 μL of Intracell Reagent A to the cell suspension. Mix well and incubate at room temperature for 15 min.
    b. Wash with 1 mL PBS +1% FBS and centrifugate for 5 min at 7000 × *g*.
    c. Remove supernatant carefully.
    d. Add 100 μL of Intracell Reagent B (permeabilization) carefully to the cell pellet, trying not to make bubbles.

Note: vortexing is strongly not recommended in this step to avoid foam.

    e. Add corresponding primary antibody to the cell suspension mix and incubate for 2 h at room temperature.
    f. Wash with 1 mL PBS +1% FBS and centrifugate at 7000 × *g*.
    g. Remove supernatant carefully.
    h. Add 100 μL of Intracell Reagent B (permeabilization) carefully to the pellet, trying not to make bubbles.
    i. Add respective secondary antibody to the cell suspension mix and incubate for 1 h in the dark at room temperature.

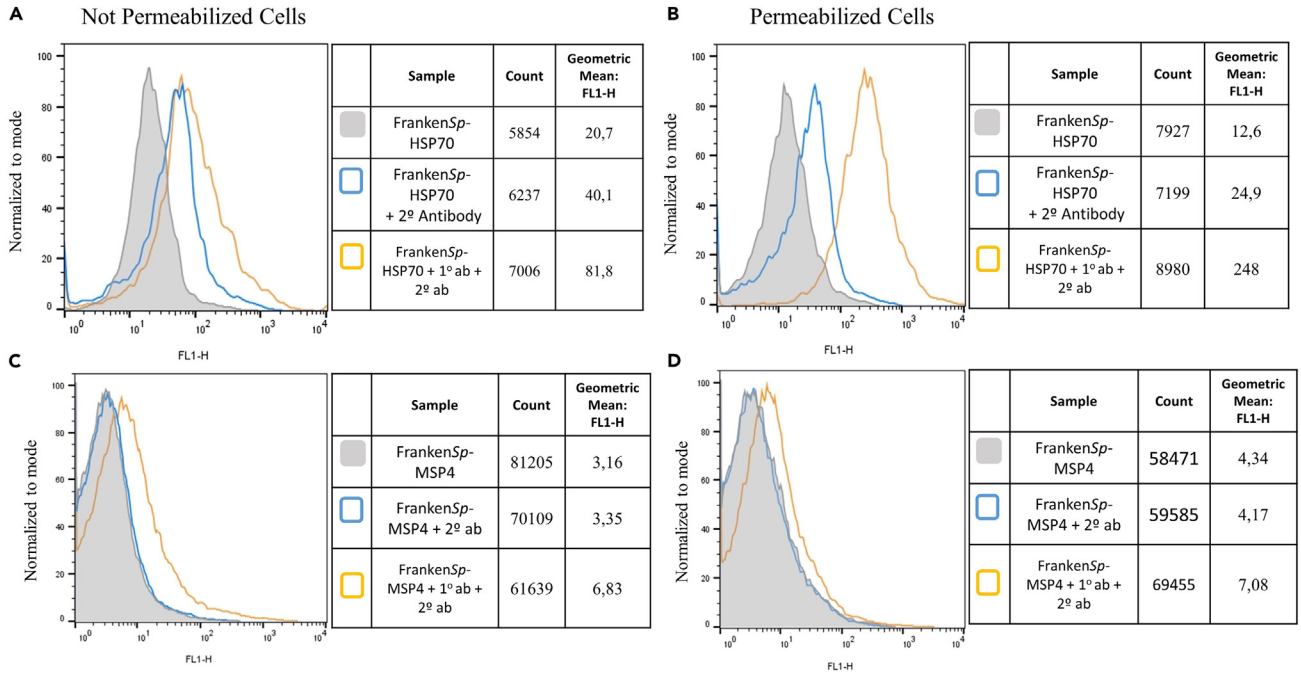

Figure 6. Flow cytometry analysis of Franken *Sphingomonas*-HSP70 and Franken *Sphingomonas*-MSP4 after 6 h of induction time with 250 μM Vanillate

Represented data have been obtained from experimental assay described by Mazuecos et al.[1] Abbreviations: ab, antibody; FrankenSp, Franken *Sphingomonas*.

⚠ CRITICAL: do not forget to include Franken *Sphingomonas* with no antibody (Franken*Sph*-MSP4 and Franken*Sph*-HSP70) and with respective only secondary antibodies as background control for cytometry analysis of permeabilized cells.

22. Wash with 1 mL PBS +1% FBS and centrifugate at 7000 × *g*.
23. Resuspend pellet with PBS +1% FBS and ensure that the cell pellet is in suspension.
24. Analyze samples using a flow cytometer (Figure 6).

Note: A FACSCalibur cytometer (BD Biosciences) was used for the flow cytometry analysis. Laser, wavelength and photomultiplier will depend on cytometer used and antibodies conjugates. In this case, we use fluorescein isothiocyanate (FITC) for excitation at 488 nm, and photomultiplier FL1.

Note: Our flow cytometry analysis revealed that HSP70 protein is predominantly produced intracellularly (Figure 6B). However, MSP4 protein is located at similar levels both inside cell and in the surface of bacterial membrane, as observed in results obtained in non-permeabilized cells (Figure 6C) and permeabilized cells (Figure 6D). It is highly recommended to add Franken *Sphingomonas*-X with no antibody incubation (grey graph) as internal control together with secondary antibody control (blue graph).

Franken *Sphingomonas*-*A. phagocytophilum* co-infection of *I. scapularis* ISE6 tick cells

Timing: 4–5 days

Here is described a protocol for co-infection analysis of Franken *Sphingomonas* with *A. phagocytophilum* in *I. scapularis* ISE6 tick cells (Figure 7). Franken *Sphingomonas*-MSP4 has

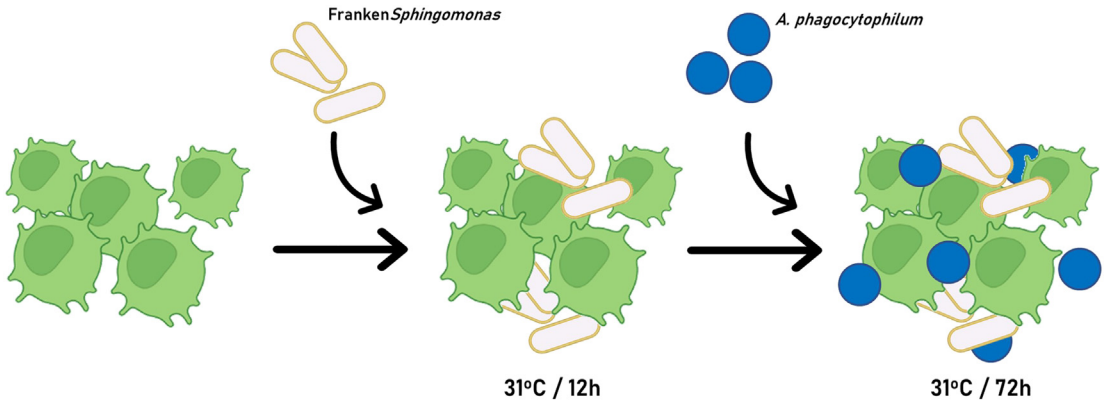

Figure 7. Representative scheme of Franken *Sphingomonas*–*A. phagocytophilum* co-infection assay in ISE6 tick cells

been reported to compete with *A. phagocytophilum* and reduce pathogen infection in both ISE6 and HL-60 cells and in *I. scapularis* ticks fed on *A. phagocytophilum*-infected C3H/HeN mice.[1] *A. phagocytophilum* infection in ISE6 tick cells is further analyzed by qPCR and confocal microscopy analysis is performed to obtain representative images of the co-infection.

25. Grow Franken *Sphingomonas* in 250 mL of R2A broth at 30°C with tetracycline (20 μg/mL) with shaking to mid-exponential phase (O.D. 600 nm = 0.6–0.8) and stimulate protein production by adding 250 μM Vanillate.
26. At the set induction time (See Step 17 and Step 18 of "characterization of Franken *Sphingomonas* by flow cytometry, centrifuge 25 mL of bacterial cultures at 4000 × *g* for 5 min.
27. Resuspend bacterial pellets in PBS +1% FBS with 250 μM Vanillate and dilute them up to O.D. 600 nm = 0.1.

Note: Calculate the final volume for resuspending the bacterial pellet (O.D. = 0.1) considering that for the co-infection assay 100 μL/mL of Franken *Sphingomonas* are needed for each replicate. For example, to make 4 replicates of each experimental condition, we need a final volume of 400 μL of Franken *Sphingomonas*-MSP4. We recommend to prepare a final volume of 0.5 mL. In case of the control Franken *Sphingomonas*-pVHD, we need 800 μL (Franken *Sphingomonas*-pVHD is used as control , see number of replicates in Figure 8). In this case, we recommend to prepare 1 mL final volume at O.D. = 0.1.

28. Culture ISE6 tick cells in L-15B300:R2A medium (1:1) and culture 500,000 cells per well in a 24 well-plate (500,000 cells in 1 mL final volume).

Note: If confocal microscopy is going to be done in parallel with co-infection assay, culture two 24-well-plates with same experimental conditions.

29. Incubate at 31°C with 100 μL/mL of Franken *Sphingomonas* or culture medium alone for control wells for 12 h.
30. Infect appropriate wells with 100 μL of semi-purified *A. phagocytophilum* (O.D. 600 nm = 0.1) or culture medium for control wells and incubate for additional 72 h at 31°C.

Note: Ensure that enough replicates and cell controls are added to the plate to perform an accurate assay. Herein, it is posted a representative plate template for co-infection assay of *A. phagocytophilum* with Franken *Sphingomonas*-pVHD, Franken *Sphingomonas*-MSP4 or Franken *Sphingomonas*-HSP70 (Figure 8).

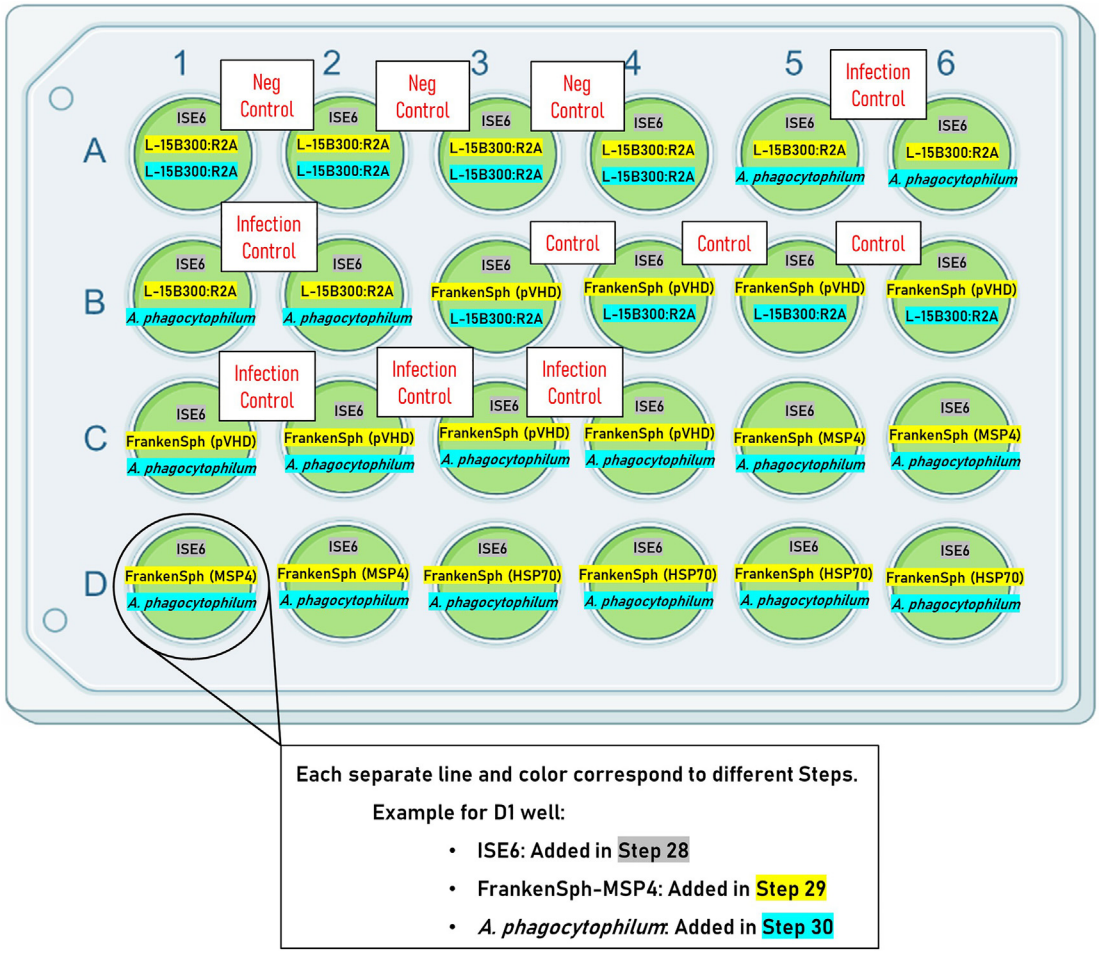

Figure 8. Representative example of a 24-wells-plate data sheet with corresponding experimental controls and experimental conditions
Below the plate, an example of a treatment has been posted for D1 well. Produced proteins or vector alone control are shown in parenthesis. Abbreviation: FrankenSph, Franken *Sphingomonas*.

⚠ CRITICAL: Even though it is not essential for analyzing the effect of Franken *Sphingomonas* during *A. phagocytophilum* infection in ISE6 cells, it is highly recommended to add replicates with ISE6 tick cells and *A. phagocytophilum* infection (without *Sphingomonas* co-culture) to determine if during this incubation time (Step 30, 72 h), it exists an accurate pathogen infection in host cells.

31. Collect cell suspension and centrifugate at 350 × *g* for 5 min to remove non-infected *A. phagocytophilum* (*Anaplasma* that have not entered to ISE6 cells during infection time). Remove supernatant.

Pause point: Cell pellet can be stored at −20°C for at least 1 week until further processing. For long-term storage, store at −80°C.

32. Extract total DNA from cell pellet by using DNeasy Blood & Tissue Kit following manufacturer's instructions (https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/dneasy-blood-and-tissue-kit).
33. Quantify DNA concentration by nanodrop or microplate reader of all samples.

34. Analyze *A. phagocytophilum* infection in ISE6 tick cells by qPCR targeting *Anaplasma msp2* gene and normalize against tick *40S ribosomal protein S4 (rpS4)* gene. Below, primers pairs and PCR reaction mix are detailed.

Primer Sequences for *msp2* and *rpS4* gene amplification

Gene	Primer pairs (F, forward; R, reverse)
msp2	F: 5′- ATGGAAGGTAGTGTTGGTTATGGTATT-3′ R:5′- TTGGTCTTGAAGCGCTCGTA-3′
rpS4	F: 5′-GGTGAAGAAGATTGTCAAGCAGAG -3′ R: 5′-TGAAGCCAGCAGGGTAGTTTG-3′

qPCR Luna Master Mix

Reagent	Amount
PCR Master Mix 2×	10 μL
DNA template (2–4 μL)	20 ng
Primer Forward (10 μM)	0.5 μL
Primer Reverse (10 μM)	0.5 μL
ddH_2O	Up to 20 μL
Final Volume	20 μL

Note: qPCR Master Mix reaction from Luna Universal qPCR Master Mix (https://international.neb.com/products/m3003-luna-universal-qpcr-master-mix#Product%20Information).

Alternative: Another adequate normalizer gene for tick cells may be *16s rDNA*.[10,11]

Characterization of Franken *Sphingomonas-A. phagocytophilum* co-infection by confocal microscopy

Timing: 3 days

Representative images of co-infection assay strongly strengthen the results obtained. Here it is described a protocol for ISE6 tick cells and Franken *Sphingomonas* localization during *A. phagocytophilum* infection.

35. After co-infection assay, collect bacterial and cell mix by pipetting up and down from 24-well plate into an Eppendorf tube.

Note: For confocal microscopy analysis, previous centrifugation to remove non-infected A. phagocytophilum as described above is optional (Step 31, "Franken *Sphingomonas-A. phagocytophilum* co-infection of *I. scapularis* ISE6 tick cells" section). If centrifugation is not performed, *A. phagocytophilum* might be observed in cell slides preparations analyzed by confocal microscopy.

36. Perform a 100 μL cytospin preparation (50,000 cells) in replicates by centrifugation at 800 × *g* for 5 min.
37. Wash with PBS-0.1% Tween 20 (PBS-T20) by immersion for 5 min.
38. Fix cells using 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature.
39. Wash three times with PBS-T20 by immersion for 5 min.
40. Permeabilized cells using 0.25% Triton for 10 min at room temperature.
41. Wash three times with PBS-T20 by immersion for 5 min.
42. Block with 1% BSA in PBS-T20 for 30 min at room temperature.

43. Incubate cell preparations with respective primary antibody diluted in 1% BSA-PBS-T20 (rabbit anti-MSP4 primary antibody and monoclonal anti-HSP70 primary antibody, 1:100) overnight at 4°C in a humidified chamber.

Note: For cell preparations with only secondary antibody (as internal control for confocal microscopy), add 1% BSA-PBS-T20 in this step.

44. Decant the solution and wash three times with PBS-T20 by immersion for 5 min.
45. Incubate cell preparations with secondary antibody in 1% BSA-PBS-T20 (1:50) using goat anti-rabbit IgG-FITC for MSP4 and goat anti-mouse IgG-FITC for HSP70.
46. Incubate for 1 h at room temperature in the dark and in humid chamber.
47. Decant secondary antibody and wash three times for 5 min by immersion in the dark.
48. Add DAPI solution (300 mM) and incubate in the dark for 5 min.
49. Add 1 drop of Prolong Diamond Antifade Mounting onto glass slide.
50. Mount coverslip and remove excess.
51. Let cell preparations dry overnight at room temperature in the dark.
52. Examine fluorescence microscopy on the following day or store at 4°C (Figure 9).

Pause point: Mounted slides can be stores at 4°C for long periods before examine under microscope.

Note: In this experimental design, it is impossible to differentiate MSP4 protein from Franken *Sphingomonas*-MSP4 or *A. phagocytophilum*, due to the use of a generic anti-MSP4 primary antibody recognizing MSP4 protein present in both bacteria. However, we can observe that most MSP4 proteins are located outside cells or around the cells, in comparison to infected ISE6 tick cells and co-infected with Franken *Sphingomonas*-pVHD or Franken *Sphingomonas*-HSP70.

EXPECTED OUTCOMES

As demonstrated by Mazuecos et al.,[1] genetic manipulation of commensal bacteria can be used to convert ectoparasite enemies into friends using a frankenbacteriosis approach (a paratransgenic approach inspired by Frankenstein and targeting tick gut microbiota involved in tick-pathogen interactions). In frankenbacteriosis, tick commensal bacteria from gut microbiota are genetically modified to produce membrane-exposed pathogen-derived proteins involved in interactions and infection of the tick and mammalian host cells.

Here, we describe a protocol for frankenbacteriosis of paratransgenic modification and validation of *Sphingomonas* bacteria transformed by electroporation with *A. phagocytophilum* genes coding for proteins MSP4 and HSP70 that reduce infection of *A. phagocytophilum* in co-culture conditions within host/vector cells. The purpose of this protocol is to generate a biotechnological tool that might mimic and compete with pathogens such as *A. phagocytophilum* both *in vivo* and *in vitro*, providing new targets and putative interventions for the control of human granulocytic anaplasmosis (HGA). In spite of the fact that we have not tested this specific protocol with other pathogens, we consider that this principle can be applied to other tick-borne diseases, or even other vector-borne diseases. As previously described,[1] co-infection assay has been tested in tick ISE6 and human HL-60 cell lines, suggesting that this protocol could be adapted to other cell lines that may be important targets for *A. phagocytophilum* and other pathogens.

In order to increase transformation efficiency and yield, and apart from all notes stated and listed under each Step in the protocol description, we recommend the use of commercial kits for isolation, purification or quantification PCR products, RNA or purified plasmids as much as possible. As assay time is several days or even weeks, and even if protocols' set up under laboratory conditions are effective, commercial kits constitute timesaving tools that guarantee technical sensitivity, a higher probability of success and reproducibility for researchers.

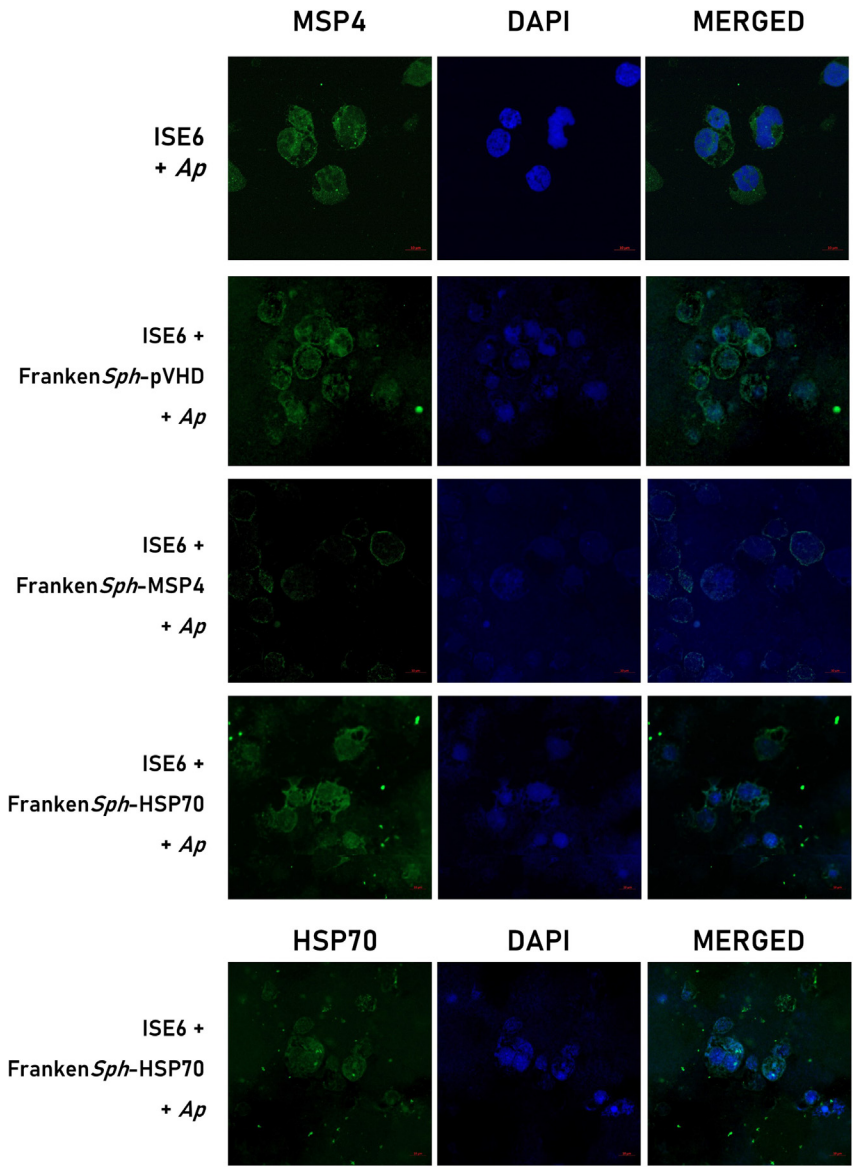

Figure 9. Representative images of immunofluorescence analysis of MSP4 and HSP70 proteins in ISE6 tick cells at 72 h after infection with *A. phagocytophilum* pathogen alone and/or in combination with Franken*Sph*-pVHD, Franken*Sph*-MSP4 and Franken*Sph*-HSP70 co-infection

Bars, 10 μm. Abbreviations, *Ap*, *A. phagocytophilum*; Franken*Sph*, Franken *Sphingomonas*.

We selected PCR techniques for quantification of *A. phagocytophilum* infection for being one of the most rigorous and sensitive tools for DNA analysis. However, other molecular techniques can be applied for infection analysis depending on biological sources available. As representative DNA analysis of co-infection assays performed under laboratory conditions, Figure 10 shows *A. phagocytophilum msp2* gene expression in human HL-60 cells (Figure 10A) and ISE6 tick cells (Figure 10B) during co-infection of *A. phagocytophilum* and Franken *Sphingomonas*-pVHD (control), Franken *Sphingomonas*-MSP4 and Franken *Sphingomonas*-HSP70. Franken *Sphingomonas*-MSP4 decreased *A. phagocytophilum msp2* DNA levels by 50% and 30% in human HL-60 cells (Figure 10A) and ISE6 (Figure 10B), respectively, in comparison to co-infection with control Franken *Sphingomonas*-pVHD.

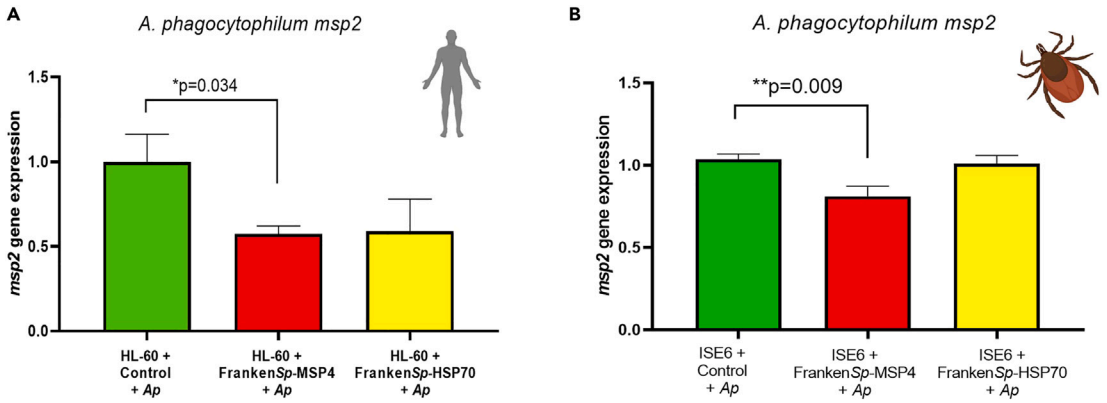

Figure 10. Detection of *A. phagocytophilum msp2* DNA levels in human HL60 and tick ISE6 cells

A. phagocytophilum msp2 DNA levels at 72 h after co-infection of (A) human HL-60 and (B) tick ISE6 cells with *A. phagocytophilum* and Franken *Sphingomonas*-pVHD (control), Franken *Sphingomonas*-MSP4 or Franken *Sphingomonas*-HSP70. DNA levels were normalized to β-*actin* and *rpS4* for HL-60 and ISE6 tick cells, respectively (Student's t-Test, *p < 0.05, **p < 0.01, n = 4 biological replicates). Data were obtained from Mazuecos et al.[1]
Abbreviations: *Ap*, *A. phagocytophilum*; Franken*Sp*, Franken *Sphingomonas*.

QUANTIFICATION AND STATISTICAL ANALYSIS

DNA levels from qPCR should be normalized against a tick normalizer gene such as *16s RNA* or *rpS4*. In this study, DNA levels are normalized using genNorm method (ddCT) as previously reported.[11,12] Normalized Ct-values can be compared between uninfected and infected tick samples by Student's t-test with equal variance ($p \leq 0.05$).

As described above, it is important to have an internal control of uninfected and infected ISE6 tick cells without Franken *Sphingomonas* co-infection, in order to ensure an optimal *A. phagocytophilum* infection during the experimental incubation for 72 h.

Franken *Sphingomonas* transformed with the expression vector, but without insert gene for expression (herein, Franken *Sphingomonas*-pVHD) must be included in the experimental design of the assay. Once *A. phagocytophilum* infection during the experimental incubation has been confirmed by qPCR, Franken Sphingomonas-inducible target protein competition with *A. phagocytophilum* during co-infection in tick cells by qPCR should be compared with Franken *Sphingomonas*-pVHD to ensure that protein target production is responsible for reducing *A. phagocytophilum* infection, and not only the presence of other bacteria.

LIMITATIONS

This protocol includes several steps that could affect the results of the experimental assay. It is important to correctly select specific plasmids and conditions for chemical or electroporation transformation, depending on bacteria genera selected. The appropriate selection of this criterion is crucial to increase yield and transformation success. Furthermore, sequencing analysis of positive transformed colonies is highly recommended in order to discard truncated or incorrect fragments insertions.

It is possible that several cell lines need specific temperature conditions for cultivation that could compromise bacterial growth. To our knowledge, *Sphingomonas* and *A. phagocytophilum* bacteria can be co-infected and cultured in a range of temperatures between 30–37°C with no limitations of the study, as has been tested for HL-60 human cells (37°C) and ISE6 tick cells (30°C). Notwithstanding, temperature and culture medium conditions should be adjusted prior to assay with a pilot study.

Effective competition of Franken *Sphingomonas*-targeted protein with *A. phagocytophilum* or another studied pathogen during co-infection in a cell line will be strongly dependent on the protein

produced by transformed bacteria. In this case, low protein expression (confirmed by flow cytometry) is enough to reduce pathogen infection in host human and tick cells by 50% and 30%, respectively. However, other pathogen infection (or the relevant protein involved in interactions during infection) may require an increase of protein production levels or bacteria concentration that generate a significant change in intracellular pathogen DNA levels to validate this approach as a promising tool for controlling infectious diseases.

TROUBLESHOOTING

Problem 1

Observation of transformants with truncated or incorrect DNA inserts is a common obstacle that affects gene expression and protein production. Although TOPO cloning reaction and Gateway cloning strategies are well-stabilized commercial solutions for optimal results and transformation success, this issue may appear under different laboratory conditions. This difficulty might appear on Step 2j, Step 6 and Step 14 from "generation of Franken *Sphingomonas* by genetic modification of *Sphingomonas* SpAR92" section.

Potential solution

- It is highly recommended to prevent plasmid recombination by using specifically designed competent cells.
- Mutations might occur during plasmid propagation in transformed cells. Selecting a sufficient number of colonies for a representative screening guarantees the transformation success. Based on our experience, picking 5–6 antibiotic-resistant colonies after each transformation should be enough to obtain at least 2 positive transformants with correct DNA inserted after validation. Genetic tools such as restriction analysis and sequencing must be done to ensure the correct direction or mutations in the target insert. If a specific mutation is detected in all the screened colonies, it might have been originated in the original plasmid or template.
- Here we provide a thorough troubleshooting guide link for cloning (https://international.neb.com/tools-and-resources/troubleshooting-guides/troubleshooting-guide-for-cloning)
- Finally, a good design of plasmids, insert and analysis of protein production must be ensured. Softwares like "addgene" or "snapgene" might help to the purpose of the assay. If protein localization is not important for the assay, protein production can be analyzed by Western-blot assay.[13]

Problem 2

No positive colonies are detected or transformants do not present the plasmid but appears with a porous membrane. Recovery time after bacterial chemical transformation or electroporation is critical for cell membrane repair, antibiotic resistance gene expression and transformation efficiency. If after colony screening *Sphingomonas* transformants do not appear after 2 h of horizontally and shaking incubation at 30°C.

Potential solution

- Use of SOC medium or fresh and equilibrated R2A medium without antibiotics, as these are optimal for cell recovery process in case of *Sphingomonas* bacteria. However, a nutrient-rich microbial broth supplement specific for the bacteria strain used may contribute to improve transformation efficiency.
- Extend recovery time from 2 h to 4 h or even let cells at 30°C horizontally with gentle shaking overnight.

Problem 3

Optimization of the Franken *Sphingomonas*-MSP4:*A. phagocytophilum* ratio to improve reduction of pathogen infection in ISE6 and HL-60 cells and in *I. scapularis* ticks fed on *A. phagocytophilum*-infected C3H/HeN mice.

Potential solution

- Perform titration experiments with different Franken *Sphingomonas*-MSP4:*A. phagocytophilum* ratios (e.g., 1:1, 1:2, 1:3, 2:1, 3:1 Franken *Sphingomonas*-MSP4:*A. phagocytophilum* ratios) in ISE6 and HL-60 cells to determine the most appropriate ratio for co-infection.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, José de la Fuente (jose_delafuente@yahoo.com).

Materials availability

There are no restrictions to the availability of newly generated materials in this study.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- All original code is available in this paper or in the original article.[1]
- Any additional information required to analyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.d.l.F.; methodology, L.M., A.G.-G., and J.d.l.F.; investigation, L.M. and A.G.-G.; writing – original draft, L.M.; revision, J.d.l.F. and L.M.; funding acquisition and supervision, J.d.l.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Mazuecos, L., Alberdi, P., Hernández-Jarguín, A., Contreras, M., Villar, M., Cabezas-Cruz, A., Simo, L., González-García, A., Díaz-Sánchez, S., Neelakanta, G., et al. (2023). Frankenbacteriosis targeting interactions between pathogen and symbiont to control infection in the tick vector. iScience106697. https://doi.org/10.1016/j.isci.2023.106697.

2. Contreras, M., Alberdi, P., Mateos-Hernández, L., Fernández de Mera, I.G., García-Pérez, A.L., Vancová, M., Villar, M., Ayllón, N., Cabezas-Cruz, A., Valdés, J.J., et al. (2017). *Anaplasma phagocytophilum* MSP4 and HSP70 Proteins Are Involved in Interactions with Host Cells during Pathogen Infection. Front. Cell. Infect. Microbiol. *7*, 307. https://doi.org/10.3389/fcimb.2017.00307.

3. Villar, M., Ayllón, N., Kocan, K.M., Bonzón-Kulichenko, E., Alberdi, P., Blouin, E.F., Weisheit, S., Mateos-Hernández, L., Cabezas-Cruz, A., Bell-Sakyi, L., et al. (2015). Identification and Characterization of *Anaplasma phagocytophilum* Proteins Involved in Infection of the Tick Vector, Ixodes scapularis. PLoS One *10*, e0137237. https://doi.org/10.1371/journal.pone.0137237.

4. Munderloh, U.G., Liu, Y., Wang, M., Chen, C., and Kurtti, T.J. (1994). Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. J. Parasitol. *80*, 533–543.

5. Munderloh, U.G., Jauron, S.D., Fingerle, V., Leitritz, L., Hayes, S.F., Hautman, J.M., Nelson, C.M., Huberty, B.W., Kurtti, T.J., Ahlstrand, G.G., et al. (1999). Invasion and Intracellular Development of the Human Granulocytic Ehrlichiosis Agent in Tick Cell Culture. J. Clin. Microbiol. *37*, 2518–2524. https://doi.org/10.1128/JCM.37.8.2518-2524.1999.

6. Kurtti, T.J., Munderloh, U.G., Andreadis, T.G., Magnarelli, L.A., and Mather, T.N. (1996). Tick cell culture isolation of an intracellular prokaryote from the tick *Ixodes scapularis*. J. Invertebr. Pathol. *67*, 318–321. https://doi.org/10.1006/jipa.1996.0050.

7. Munderloh, U.G., and Kurtti, T.J. (1989). Formulation of medium for tick cell culture. Exp. Appl. Acarol. *7*, 219–229. https://doi.org/10.1007/BF01194061.

8. de la Fuente, J., Ayoubi, P., Blouin, E.F., Almazán, C., Naranjo, V., and Kocan, K.M. (2005). Gene expression profiling of human promyelocytic cells in response to infection with *Anaplasma phagocytophilum*. Cell Microbiol. *7*, 549–559. https://doi.org/10.1111/j.1462-5822.2004.00485.x.

9. Saito, M., Ikunaga, Y., Ohta, H., and Kurusu, Y. (2006). Genetic Transformation System for Members of the Genera, *Sphingomonas, Sphingobium, Novosphingobium* and *Sphingopyxis*. Microbes Environ. *21*, 235–239. https://doi.org/10.1264/jsme2.21.235.

10. Ayllón, N., Villar, M., Busby, A.T., Kocan, K.M., Blouin, E.F., Bonzón-Kulichenko, E., Galindo,

R.C., Mangold, A.J., Alberdi, P., Pérez de la Lastra, J.M., et al. (2013). *Anaplasma phagocytophilum* inhibits apoptosis and promotes cytoskeleton rearrangement for infection of tick cells. Infect. Immun. *81*, 2415–2425. https://doi.org/10.1128/IAI.00194-13.

11. Zivkovic, Z., Blouin, E.F., Manzano-Roman, R., Almazán, C., Naranjo, V., Massung, R.F., Jongejan, F., Kocan, K.M., and de la Fuente, J. (2009). *Anaplasma phagocytophilum* and *Anaplasma marginale* elicit different gene expression responses in cultured tick cells. Comp. Funct. Genomics *2009*, 705034. https://doi.org/10.1155/2009/705034.

12. Koci, J., Simo, L., and Park, Y. (2013). Validation of internal reference genes for real-time quantitative polymerase chain reaction studies in the tick, *Ixodes scapularis* (Acari: Ixodidae). J. Med. Entomol. *50*, 79–84. https://doi.org/10.1603/me12034.

13. Almazán, C., Moreno-Cantú, O., Moreno-Cid, J.A., Galindo, R.C., Canales, M., Villar, M., and de la Fuente, J. (2012). Control of tick infestations in cattle vaccinated with bacterial membranes containing surface-exposed tick protective antigens. Vaccine *30*, 265–272. https://doi.org/10.1016/j.vaccine.2011.10.102.