AMB Express

A method for creating in-frame insertions of fluorescent proteins in non-model Gram-negative bacteria

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Supplementary Table S1: Triparental mating of suicide/endonuclease plasmids

Day 1

1. Collect 1 ml of each culture (donor, helper, recipient) in sterile labelled microcentrifuge tubes, and centrifuge at 6000 x g for 1 minute
2. Discard the supernatants, and add 500 µl of fresh LB to each tube
3. Resuspend the cells, and centrifuge at 6000 x g for 1 minute to remove traces of antibiotics
4. Repeat steps 2 and 3 once more
5. Add 100 µl of fresh LB to each tube, and resuspend the cells
6. Combine all 3 types of cells into a single microcentrifuge tube, and centrifuge at 6000 x g for 1 minute
7. Resuspend the cell mixture in a total of 50–80 µl of fresh LB, and vortex the cell suspension vigourously for 30 seconds
8. Using aseptic technique, carefully place a strip of sterile nitrocellulose membrane on a non-selective LB agar plate
9. Pipette out 50 µl of the triparental mating mixture onto this membrane, and allow the spot to dry
10. Incubate the plate upright (i.e., bacteria facing up) at 37°C overnight to allow for transfer of the plasmid

Day 2

1. Add 500 µl of fresh LB to a sterile labelled microcentrifuge tube
2. Using aseptic technique, carefully transfer the nitrocellulose membrane containing the bacteria to the microcentrifuge tube
3. Vortex vigourously to dislodge the attached cells into the medium
4. Plate 100 µl of the cell mixture on 2 of the selective PIA plates
5. Centrifuge the remaining cell mixture at 6000 x g for 1 minute, then resuspend the cells in 100 µl of fresh LB
6. Plate the remaining cells on the final selective PIA plate
7. Incubate the plates for up to 48 hours or until screenable colonies are obtained

Supplemetary Table S2: Preparation of electrocompetent cells and electroporation of endonuclease plasmid

1. Transfer 50 ml of fresh LB-Lennox to a 250 ml Erlenmeyer culture flask supplemented with 10 mM $MgSO_4.6H_2O$
2. Back-dilute (1:50) the overnight culture into flask, and incubate at 30°C and 200 rpm until OD_{600} reaches 1.0 – this should take approximately 3–4 hours
3. While the culture is growing, place a 50 ml Falcon tube on ice to cool it down
4. Once the optimum OD_{600} has been reached, transfer the culture to the pre-chilled Falcon tube, and place on ice for a further 30 minutes, swirling occasionally to prevent the cells from settling
5. Centrifuge the culture at 4000 x g at 4°C for 10 minutes
6. Discard the supernatant, and add 10 ml of the sterile glycerol solution
7. Resuspend the pellet using a pipette (do *not* vortex the cells), then add a further 40 ml of the sterile glycerol solution
8. Repeat steps 5–7 once more
9. Centrifuge the culture at 4000 x g at 4°C for 10 minutes
10. Discard the supernatant, and add 1–2 ml of the sterile glycerol solution
11. Resuspend the cells using a pipette, and make 50 µl aliquots of the cell suspension
12. Store the aliquots at –80°C until required
13. For each electroporation reaction, place 1 aliquot of *S. maltophilia* electrocompetent cells and 1 electroporation cuvette (0.1 cm gap) on ice
14. To each aliquot, add 100–200 ng of pDAI-SceI-SacB and transfer the electroporation mixture to the corresponding cuvette
15. Place the cuvettes on ice for a further 2 minutes
16. Electroporate the cuvettes with the following conditions – 25 kV/cm field strength, 25 µF capacitance, and 200 Ω resistance – ensuring that the time constant is at least 5.0 ms
17. Immediately add 900 µl of SOC medium to the cuvettes, and pipette up and down to suspend all the cells
18. Transfer the cells to sterile labelled microcentrifuge tubes, and recover for 3 hours at 30°C and 200 rpm
19. Plate 100 µl of the cell mixture on 2 of the selective LB plates
20. Centrifuge the remaining cell mixture at 6000 x g for 1 minute, then resuspend the cells in 100 µl of fresh LB
21. Plate the remaining cells on the final selective LB plate
22. Incubate the plates at 30°C for up to 48 hours or until screenable colonies are obtained

Supplementary Table S3: Colony PCR protocol

1. In a sterile hood, prepare and label as many 0.5 ml microcentrtifuge tubes as required
2. Add 80–100 µl of sterile MilliQ water to each tube
3. With sterile pipette tips, carefully scrape a small portion of each colony and transfer to the corresponding tube
4. Vortex vigourously for 10–20 seconds and use 2 µl as DNA template per 25 µl PCR reaction

Supplementary Table S4: List of screening primers used

Type of primer	Primer sequence (5' → 3')
egfp gene-specific	FP: CACATGAAGCAGCACGACTT RP: TGCTCAGGTAGTGGTTGTCG
atpG gene-specific	FP: TGTGCTTTGAACGAACGCGG RP:TGATTTCGCGTCCGCTTGCCAT
smlt1054 gene-specific	FP: CATTGATGGTGATGCCGCG RP: AGACGCTCTGTTGAACCTGG
smlt0387 gene-specific	FP: ATGAAGAATTCGCTGATTGCTCTGG RP: GCGGAATCCAATTCGTTGCAAT

Supplementary Table S5: Sequences of homology regions used for insertions and replacement

***smlt1054:egfp* (1107786–1108271, minus-strand)**

Upstream homology sequence (1107585–1107785, plus-strand)
CGCGCCTGCGGCGCTGTTGGCGAGGGCCTGCTGGTGCGCGCGCTGGACTGCATCGGTGCGGGCCTCCTGCTGCGC
TGCCCGGGTGGTGGCAACGGCAAGGTCCGCAGTGCGGTCGCGCAGGGTCCATCCCAGCCAGGCGCAGCTCGCGTG
GCTACCGGCAAGGACCAGCAACCCGATGCCCAACCTCAGTCCCGCGGGCG

Downstream homology sequence (1107788–1108088, plus-strand)
CTGCAGGGCTCCGCCGGCGGCACGGTACGCCGCGCGCAGTGTTTCCAGTGCGTGCTCCTTCTGGCCGTAGCCGGC
GCCCGGCAGCGATGCCCAGATGCGCCGGGCCGCGGTGACGGCGGCATCGAAGCGTCCCAGCCGGATCAGCTCGTA
GGCACCGCACTGTTTCAGCAGGGCGACTGCTGCACGGTCCTGGGAGACTGGCCCGAAGTCAGGCAGACCCAGTCG
CGCGCGCAGGTCATCCCAGGTGCTGCGCAGGAACTGGTAGCGGCCGGCGGCGCTGGATTTGATGCCATAGCGTGG

Linker + eGFP sequence (reverse complement)
TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCG
CTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTC
GCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAA
GTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT
GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTC
GAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTC
GGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGT
CAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCC
GTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAG
GATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGCTGCCGCTGCCGCTGCC

***atpG:egfp* (4219920–4220783, minus-strand)**

Upstream homology sequence (4219080–4219919, plus-strand)
ACGGCATGCGGCCCAGCAGTGCCGACACTTCGGTACCGGCCAGGGTGTAGCGGTAGATGTTGTCGACGAACAGCA
GGACGTCCTTGCCCTTGCCGTTTTCGTCCTTCTCGTCGCGGAAGTACTCGGCCATGGTCAGGCCGGTCAGGGCGA
CGCGCAGACGGTTGCCCGGCGGCTCGTTCATCTGGCCGTACACCATCGCCACCTTGTCGAGGACGTTGGAGTCCT
TCATTTCGTGGTAGAAGTCGTTGCCCTCACGGGTACGCTCACCCACGCCGGCGAACACGGACAGACCGCTGTGCG
CCTTGGCGATGTTGTTGATCAGCTCCATCATGTTGACGGTCTTGCCGACGCCGGCGCCGCCGAACAGGCCGACCT
TGCCGCCCTTGGCGAACGGGCACATCAGGTCGATGACCTTGATGCCGGTTTCCAGCAGTTCGGTGGCCGGGGACT
GGTCTTCGTACGACGGGGCCGCACGGTGGATTTCCCAGCTGTCGCTGGCGGCCACCGGGCCGGCTTCGTCGATCG
GACGGCCGAGCACGTCCATGATGCGGCCCAGGGTGCCGGCGCCGACCGGCACCGAGATGCCACGGCCGGTGTTGA
CGGCAACCAGGTTGCGCTTCAGGCCGTCGGTGGAACCGAGGGCGATGGTACGCACCACGCCGTCGCCCAGCTGCT
GCTGGACTTCGAGGGTGATCTCGGTGTTTTCCACCTTCAGTGCGTCGTACACCTTCGGCACCGATTCACGCGGGA
ATTCGACGTCGACGACCGCGCCGATGATCTGAACGATCTTGCCCTGACTCATTGCTGCATCCTCTAATGTGTGCT
TTGAACGAACGCGG

Downstream homology sequence (4219917–4220783, plus-strand)
GACTGCTGCCGCGCCGCCGACGATTTCGGAGATTTCCTGGGTGATCGCTGCCTGGCGCGCCTTGTTGTAGACGAG
CTGCAGGGTGCCGATCAGCTTGTTGGCGTTGTCGCTCGCCGCCTTCATCGCAACCATGCGTGCCGCATGCTCGGA
GGCGACGTTTTCCAGCAGTGCCTGGTACACCAGCGACTCGATGTAACGCGTCATCACGTGCTCGAGCACGGTCGC
GGCATCGGGTTCGTACAGGTAATCCCAGTCGTGGTGAGCGACCTGCTTCTCAGCCGGCGGCAGCGGCAGCAGCTG
GTCGAAGCTGGCCTTCTGCACCATGGTGTTCACGAAGCGGTTGTAGACCAGGTACACGCGGTCGATCTTGCCTTC
GGTGAAGGCGTCGAGCATGACCTTGATCACACCGATCAGCGATTCCAGCTTCGGCACATCGCCGATGTGGGTCAC
GCTGCCGACCATGTTGACCTTCACGCGGCGGAAGAAGGTCGAAGCCTTCTGGCCGATGGTCACCAGGTCCACTTC
CGCACCCTTGTCCTGCCATGCCTTGGCTTCGCCCAGCATCTTGCGGAACAGGTTGTTGTTCAGGCCGCCGGCCAG
GCCGCGATCGGAGGAGATCACGATGAAGCCGACCCGCTTGACCTGCTCGCGCTCGACCAGGAACGGATGCTGGTA
GTCGGTGCTGGCCTGGGCCAGGTGACCGATCACCTGCTTCATCGCCTGCGCGTACGGACGCGAGGTCTTCATCCG
ATCCTGCGCCTTGCGGATCTTGGAGGCCGAGACCATTTCCAGGGCGCGCGTCACCTTGCGGGTGTTCTGCACGCT
CTTGATCTTGGTTTTGATTTCGCGTCCGCTTGCCAT

Linker + eGFP sequence (reverse complement)

TTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCG
CTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTC
GCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAA
GTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTGTACTCCAGCTT
GTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTC
GAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTC
GGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGTAGGT
CAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCC
GTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAG
GATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGCTGCCGCTGCCGCTGCC

***smlt0387:mCherry* (384354–384926, plus-strand)**

Upstream homology sequence (384021–384412, plus-strand)

CACGCGTTCGGCGCGCTGGTAGTCGGCCGCCTGCAGCGCGTGTGCGGTGTGTGGCGACAAGAGGGCAACGCATGC
AAAGACAGCGGCGCGGACACACGTCCGCAGCGGATCGGAAACGAGCAAGGCAACCTCCTGTTGCAATCGTCGAAG
GATTACAGATGCACGCGCGCCGCGCGCGTATGCCAGACGGAGCCTGAATCAAGCCCAAAAATTCAGCAATGCGAA
GACCACTGTTTTCAGGGAGCGTTGTGGCGAATAACGGAGAATTTACATTCCCGATGCGATGGCGCCCTGGCGCTG
CTGCACACCATAAGAAAGTAAAGGTGTACCCCCATGAAGAATTCGCTGATTGCTCTGGCCCTGGCCGCTGCCCTG
CCGTTCACCGCTTCGGCT

Downstream homology sequence (384927–385427, plus-strand)

TTGCTTCCTCGGCGCGCCTGCGCGCTGAAGATGCCTCGACAGGCCCGGCCCATGCCGGGCCTGTTGCGTTTCGGG
CTGGGCAAACGCTACCTGACCGTGTTGAGGTGGACGGGCGGCATTGCAACGAATTGGATTCCGCAGGCGTCGGCT
GCGCCCTAGGGTGAGTGGCCTGTCCTGCAGGAATGCAACGATGACCCCGTTTCCCTCGCCCCGCGATTCTCCCTG
CCGCTTGTTGCTGCTGGATCCGCATCCACTGTTGCGGCATGGCGTGCAGGAACTGCTGGCCCGGCAGGCGGGCCT
GCAGATCGATGGAAGCTATGGCCGCAGCGGTGATCTGCTGCAGCGCCTGCAGCAGCAGGCAGCTGCGATCGATCT
GCTGCTGGTTGACGCCTTGCCGATCGGTGACAGCGGGGAAGGCCTGGAACTGCTGCGGCACGTGTCACGACATTG
GCCGGCAGTGCCGATCCTGGTGCTGTCCGCACACTGCAACGCGGGTGTCG

Replacement gene (mCherry sequence + linker + smlt0387)

ATGAAGAATTCGCTGATTGCTCTGGCCCTGGCCGCTGCCCTGCCGTTCACCGCTTCGGCTATGGTGAGCAAGGGC
GAGGAGGATAACATGGCCATCATCAAGGAGTTCATGCGCTTCAAGGTGCACATGGAGGGCTCCGTGAACGGCCAC
GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCACCCAGACCGCCAAGCTGAAGGTGACCAAG
GGTGGCCCCCTGCCCTTCGCCTGGGACATCCTGTCCCCTCAGTTCATGTACGGCTCCAAGGCCTACGTGAAGCAC
CCCGCCGACATCCCCGACTACTTGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAG
GACGGCGGCGTGGTGACCGTGACCCAGGACTCCTCCCTGCAGGACGGCGAGTTCATCTACAAGGTGAAGCTGCGC
GGCACCAACTTCCCCTCCGACGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCTCCGAGCGGATG
TACCCCGAGGACGGCGCCCTGAAGGGCGAGATCAAGCAGAGGCTGAAGCTGAAGGACGGCGGCCACTACGACGCT
GAGGTCAAGACCACCTACAAGGCCAAGAAGCCCGTGCAGCTGCCCGGCGCCTACAACGTCAACATCAAGTTGGAC
ATCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAACGCGCCGAGGGCCGCCACTCCACCGGCGGC
ATGGACGAGCTGTACAAGGGCAGCGGCAGCGGCAGCGCTGAGAACCTGTCGTACAACTACGCTGAAGCCGACTAC
GCCAAGACCGACGTCGATGGCATCAAGGCTGACGGCTGGGGCGTCAAGGGTTCCTACGGCTTCCTGCCGAACTTC
CACGCGTTCGGTGAATACAGCCGTCAGGAAGTCGACCACACCAACATCAAGGTTGACCAGTGGAAGGTCGGTGCC
GGCTACAACGTCGAAATCGCTCCGTCGACCGACTTCGTTGCCCGCGTTGCCTACCAGAAGTTCGATCGCAAGCAC
GGCCTGGACTTCAACGGCTACAGCGCTGAAGCCGGTATCCGCACCGCCTTCGGTGCCCACGCCGAGGTCTACGGC
ATGGTCGGCTACGAAGACTACGCCAAGAAGCACGGCGTCGACATCGACGGCCAGTGGTACGGCCGCCTGGGTGGT
CAGGTCAAGCTGAACCAGAACTGGGGCCTGAACGGCGAGCTGAAGATGAACCGCCACGGCGACAAGGAATACACC
GTCGGCCCGCGCTTCAGCTGGTAA

Note: All genomic coordinates are with reference to the genome of strain K279a – partial genomic data for strain 44/98 is available, but not published.

Supplementary Information S6: Common problems and troubleshooting

Problem	Possible cause	Possible solution(s)
No/few colonies were obtained after triparental mating	Incorrect *E. coli* host was used to maintain the pGPI-SceI-XCm	Maintain pGPI-SceI-XCm in a λ *pir*$^{+}$ strain
	Incorrect antibiotic selection in *E. coli* overnight culture	Use Tp (50 μg/ml) or Cm (20 50 μg/ml) for pGPI-SceI-XCm, Tc (10 μg/ml) for pDAI-SceI-SacB
	Carry-over of antibiotics during triparental mating	Properly discard supernatants during washing steps, wash cells an additional time
	Incorrect ratio of D/H/R cells in triparental mating mixture	Change ratio of D/H/R cells (successful combinations include 3:3:1 and 5:5:1)
	Incorrect antibiotic selection on PIA plates	Decrease amounts of Tp and Cm (ex-conjugants) or Tc (mutants), decrease amount of Nor for counter-selection
	Insufficient incubation time	Incubate plates for up to 72 h, incubate plates at 37°C
	Target gene is essential	Interfering with function could cause a lethal phenotype, mutation might not be possible
E. coli colonies obtained after triparental mating	Insufficient antibiotic counter-selection	Use more Nor for counter-selection, use another antibiotic to which *E. coli* is sensitive but *S. maltophilia* is resistant
No/few colonies were obtained after electroporation of the endonuclease plasmid	Cells were not electrocompetent enough	Prepare fresh electrocompetent cells, reduce field strength to 18–20 kV/cm
	Insufficient recovery post-electroporation	Recover cells in SOC medium for 3 hours
	Incorrect antibiotic selection on LB plates	Decrease amount of Tc for selection
	Insufficient incubation time	Incubate plates for up to 72 h, incubate plates at 37°C
Few true transformants obtained after electroporation of endonuclease plasmid	Insufficient amount of plasmid used for transformation	Increase amount of plasmid used in electroporation reaction, supplement LB with PMBN (5–10 μg/ml) during back-dilution to increase membrane permeability
	Insufficient recovery post-electroporation	Recover cells in SOC medium for 3 hours
	Incorrect antibiotic selection on LB plates	Increase amount of Tc for selection
	Insufficient incubation time	Incubate plates for up to 72 h, incubate plates at 37°C

Note: Tc is stable at 37°C for a maximum of 3 days – if plates need to be incubated for longer, incubate at room temperature or 28–30°C.

Supplementary Figure S1: Additional microscopy images

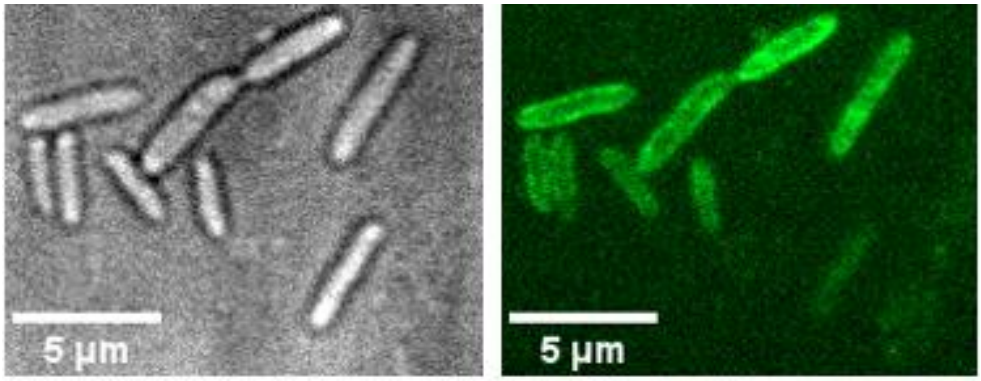

Figure S1.1. Transmission (left) and fluorescence (right) microscopy of *S. maltophilia* expressing *atpG:egfp*.

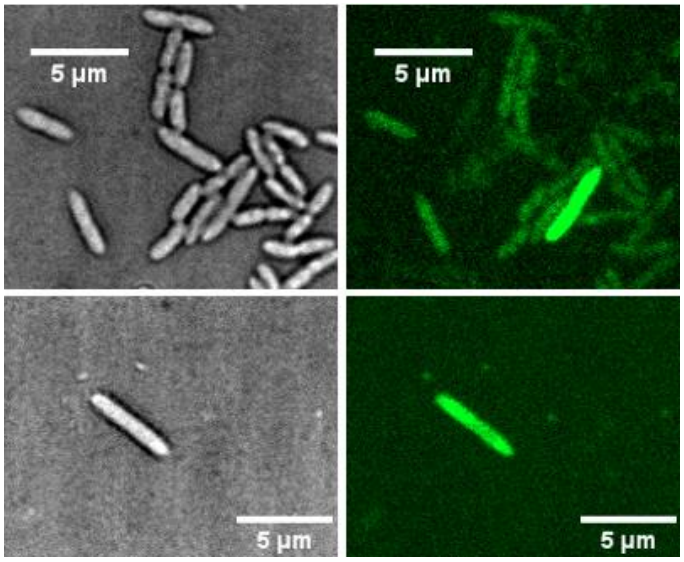

Figure S1.2. Transmission (left) and fluorescence (right) microscopy of *S. maltophilia* expressing *smlt1054:egfp*.

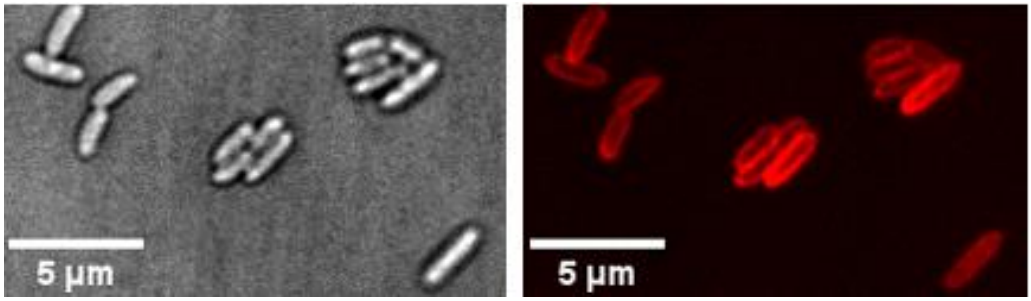

Figure S1.3. Transmission (left) and fluorescence (right) microscopy of *S. maltophilia* expressing *smlt0387:mCherry*.