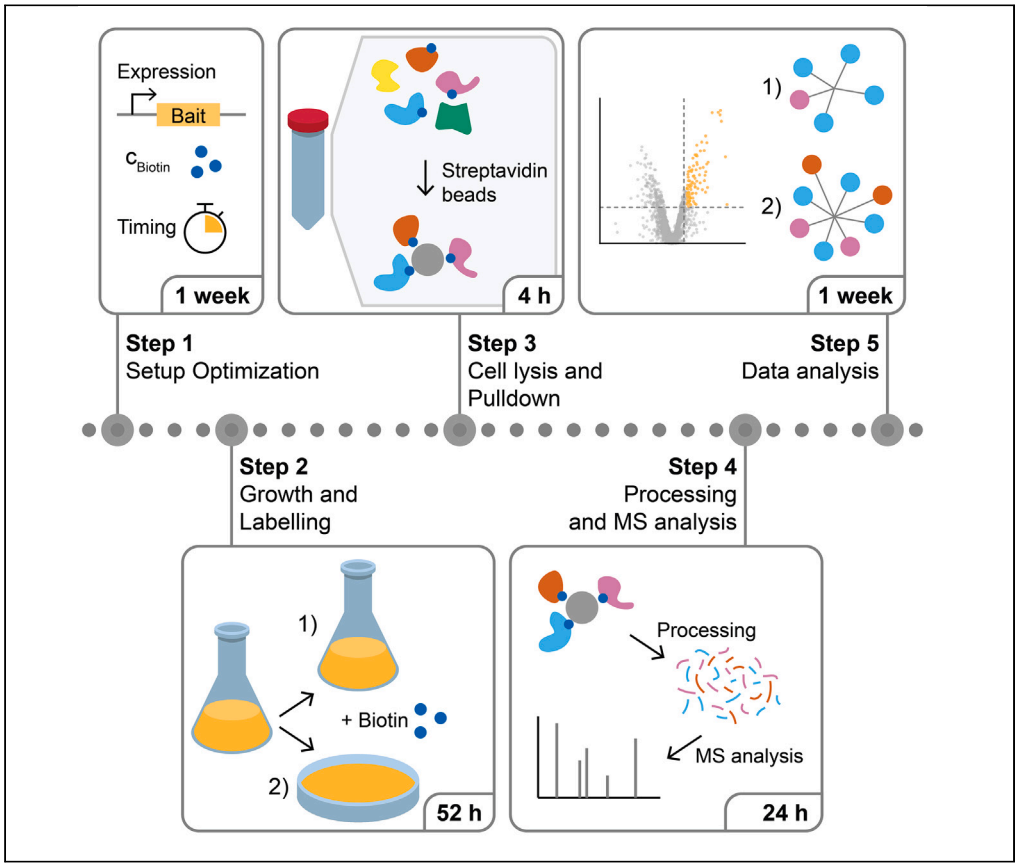
STAR Protocols

CellPress
OPEN ACCESS

Protocol

A miniTurbo-based proximity labeling protocol to identify conditional protein interactomes *in vivo* in *Myxococcus xanthus*



Protein-protein interactions are foundational for many cellular processes. Such interactions are especially challenging to identify if they are transient or depend on environmental conditions. This protocol details steps to identify stable and transient protein interactomes in the bacterium *Myxococcus xanthus* using biotin ligase miniTurbo-based proximity labeling. We include instructions for optimizing the expression of control proteins, *in vivo* biotin labeling of bacteria grown on a surface or in suspension culture, enrichment of biotinylated proteins, and sample processing for proteomic analysis.

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

Marco Herfurth, Franziska Müller, Lotte Søgaard-Andersen, Timo Glatter

marco.herfurth@mpi-marburg.mpg.de (M.H.)
franziska.muller@mpi-marburg.mpg.de (F.M.)
timo.glatter@mpi-marburg.mpg.de (T.G.)

Highlights

Optimization of miniTurbo-based biotin proximity labeling *in vivo*

Step-by-step protocol from protein design to identification of conditional interactomes

In vivo biotinylation and quantitative shotgun proteomics

Detection of the interactome of the cell polarity regulator and small GTPase MglA

Herfurth et al., STAR Protocols 4, 102657
December 15, 2023
https://doi.org/10.1016/j.xpro.2023.102657

STAR Protocols

CellPress
OPEN ACCESS

Protocol

A miniTurbo-based proximity labeling protocol to identify conditional protein interactomes *in vivo* in *Myxococcus xanthus*

Marco Herfurth,[1,3,4,*] Franziska Müller,[1,3,4,*] Lotte Søgaard-Andersen,[1] and Timo Glatter[2,3,4,5,*]

[1]Department for Ecophysiology, Max-Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany

[2]Facility for Proteomics and Mass Spectrometry, Max-Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: marco.herfurth@mpi-marburg.mpg.de (M.H.), franziska.muller@mpi-marburg.mpg.de (F.M.), timo.glatter@mpi-marburg.mpg.de (T.G.)
https://doi.org/10.1016/j.xpro.2023.102657

SUMMARY

Protein-protein interactions are foundational for many cellular processes. Such interactions are especially challenging to identify if they are transient or depend on environmental conditions. This protocol details steps to identify stable and transient protein interactomes in the bacterium *Myxococcus xanthus* using biotin ligase miniTurbo-based proximity labeling. We include instructions for optimizing the expression of control proteins, *in vivo* biotin labeling of bacteria grown on a surface or in suspension culture, enrichment of biotinylated proteins, and sample processing for proteomic analysis.

For complete details on the use and execution of this protocol, please refer to Branon et al. (2018).[1]

BEFORE YOU BEGIN

This protocol describes the steps for conducting biotin ligase-based proximity labeling in live *Myxococcus xanthus* cells using miniTurbo (mTurbo),[1] followed by shotgun proteomics mass spectrometry to identify protein interactomes. In this method, the protein of interest, or bait, is fused to mTurbo, which catalyzes the formation of a highly reactive but short-lived biotinoyl-5′-AMP intermediate from biotin and ATP. This intermediate then diffuses out of the active site of the enzyme and covalently couples biotin to amine groups in Lys residues within a distance of a few nm^2. Therefore, only proteins that are in close proximity to the tagged bait protein are biotinylated.[2] Subsequently, biotinylated proteins are purified using magnetic Streptavidin affinity beads (Strep beads) and identified using mass spectrometry. A distinct advantage of mTurbo-based proximity labeling is that the highly reactive biotinoyl-5′-AMP intermediate covalently couples biotin to proteins in proximity of the bait, and, therefore, not only allows to identify stable interaction partners but also low-affinity, transiently interacting protein partners.

We describe the general steps for applying mTurbo-based proximity labeling in bacteria by identifying the proximity interaction network of the Ras-like GTPase MglA, which controls the cell polarity of *M. xanthus* cells.[3–5] The rod-shaped *M. xanthus* cell translocates on surfaces with defined leading and lagging cell poles.[5] MglA is the key regulator of motility and localizes to and defines the leading cell pole in its active GTP-bound state.[3,5,6] At this pole, MglA interacts with downstream effector proteins to activate two different motility systems.[7–11] Occasionally, the

localization of MglA switches to the opposite pole resulting in an inversion of the direction of cell motility.[3–5] The mechanisms underlying the dynamic polar localization of MglA as well as the downstream effectors of MglA are well-studied and offer a good foundation to establish and benchmark mTurbo-based proximity labeling in *M. xanthus*.[3,6,8,11–16] We recommend to establish, adapt and optimize this protocol to different bacterial systems by using a bait protein with established and verified protein interaction partners, ideally including direct and/or indirect as well as conditional interactions. Also, using MglA as a test bait allows studying conditional interactomes. Specifically, the two motility systems of *M. xanthus*, type IVa pili (T4aP)-dependent motility and gliding motility rely on surface contact to translocate cells.[4] Furthermore, surface contact was reported to activate proteins that regulate *M. xanthus* polarity,[17] suggesting that surface contact is important for the activation and/or regulation of the two motility systems, and that MglA might potentially have different interactomes depending on whether *M. xanthus* cells are grown on a surface or in suspension culture.

To obtain optimal results and reduce the non-specific background, it is important to consider that the mTurbo bait protein (in our case MglA-mTurbo-FLAG) is active and expressed at or close to native levels. Moreover, it is important to include a control protein (here a sfGFP-mTurbo-FLAG fusion) that localizes to the same subcellular compartment as the bait to reduce the non-specific background and increase specificity in the proteomics data. Additionally, the bait and control protein should be tagged (i.e., FLAG-tag, Myc-Tag, etc.) to be able to assess the expression and stability of the bait and control protein fusions using western blotting.

In *M. xanthus,* the gene encoding the MglA-mTurbo-FLAG is integrated by double homologous recombination at the native site to replace the wild-type allele, offering high strain stability and native expression levels of the bait protein. The control protein is expressed from a plasmid integrated in a single copy by site specific recombination into the genome and under the control of an inducible promoter, allowing the adjustment of the expression level to that of the bait protein.

Preparing strains containing mTurbo fusions

Timing: 2–3 weeks

The main preparation to start proximity labeling is the design and generation of bacterial strains expressing the bait protein fused to mTurbo (MglA-mTurbo-FLAG), as well as a matching control protein fusion (sfGFP-mTurbo-FLAG).

1. Generate appropriate plasmids.

   Note: Standard cloning methods were used to generate plasmids expressing the bait protein fusion and the control protein under control of an inducible promoter. Cloning methods, plasmid backbones and inducible promoters vary strongly between bacteria and have to be chosen accordingly.

2. Transform and verify strains.

   Note: Transformation and verification of strains depend on the organism and the used backbones. We routinely use electroporation as a method for transformation and double homologous recombination to integrate the construct encoding the bait-mTurbo fusion and site specific recombination to integrate the plasmid encoding the control protein.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
α-FLAG (1:2,000)	Rockland	600-401-383
α-LonD (1:5,000)	Treuner-Lange et al.[18]	N/A
Goat α-rabbit immunoglobulin G peroxidase conjugate (1:15,000)	Sigma	A0545-1ML
Bacterial and virus strains		
Myxococcus xanthus DK1622	Kaiser et al.[19]	DK1622
M. xanthus mglA::*mglA-miniTurbo-FLAG*	This study	SA12042
M. xanthus 18–19::P_{van} *sfGFP-miniTurbo-FLAG*	This study	SA12027
Chemicals, peptides, and recombinant proteins		
Biotin	Thermo Fisher Scientific	29129
Streptavidin-HRP (1:4,000)	Bio-Rad	STAR5B
Vanillate	Sigma-Aldrich	H36001
Gentamycin sulfate	Carl Roth	0233.4
Kanamycin sulfate	Carl Roth	T832.4
Ammonium bicarbonate	Sigma-Aldrich	A6141
Sodium dodecyl sulfate	Carl Roth	CN30.3
Sodium deoxycholate	Sigma-Aldrich	D6750
Triton X-100	Carl Roth	3051.2
Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP)	Sigma-Aldrich	75259
Iodoacetamide	Sigma-Aldrich	I1149
Trypsin – sequencing grade modified	Promega	V5111
Acetonitrile, LC-MS grade	Thermo Fisher Scientific	51101
Trifluoroacetic acid	Thermo Fisher Scientific	85183
Water, LC-MS grade	Thermo Fisher Scientific	51140
Formic acid, LC-MS grade	Thermo Fisher Scientific	85178
Chromabond C18 spin columns	Macherey-Nagel	730522.25
Acclaim PepMap100 trap column C18 5 µm, 0.5 × 5 mm	Thermo Fisher Scientific	160454
ReproSil-Pur 120 C18-AQ, 2.4 µm	Dr. Maisch	r124.aq.0001
Critical commercial assays		
660 nm protein assay	Pierce	22662
G25 desalting columns	Cytiva	28918007
Streptavidin magnetic beads	Pierce	88817
cOmplete protease inhibitor cocktail	Roche	11836145001
4%–20% Mini-PROTEAN TGX PAGE gel	Bio-Rad	4561093
Restore PLUS western blot stripping buffer	Thermo Scientific	46430
Recombinant DNA		
miniTurbo	Branon et al.[1]	N/A
pMH97 (P_{van} *sfGFP-miniTurbo-FLAG*)	This study	N/A
pMP180 (integration of *mglA-miniTurbo-FLAG*)	This study	N/A
Software and algorithms		
MaxQuant	https://www.maxquant.org/	Version 2.0.3.0 and higher
Perseus	https://maxquant.net/perseus/	Version 1.6.10.43 and higher

MATERIALS AND EQUIPMENT

TPM buffer

Reagent	Final concentration
Tris-HCl pH 7.6	10 mM
Potassium phosphate pH 7.6	1 mM
$MgSO_4$	8 mM
ddH_2O	

Stable for several months at room temperature (RT).

Alternatives: Other buffers such as PBS are also appropriate for washing cells and can be adjusted to the organism used.

RIPA lysis buffer	
Reagent	Final concentration
Tris-HCl pH 7.0	50 mM
NaCl	150 mM
Triton X-100	1% (vol/vol)
Sodium deoxycholate (SDC)	0.5% (wt/vol)
Sodium dodecyl sulfate (SDS)	0.2% (wt/vol)
ddH_2O	
Store at 4°C for up to a year.	

⚠ CRITICAL: Contact with Triton X-100, SDS and SDC cause skin and eye irradiation. Wear protective gloves/protective clothing/eye protection/face protection. Do not inhale SDS.

Alternatives: Different lysis buffer formulations may be adjusted to your organism. Adjusting NaCl or detergent concentrations may impact the stringency of the streptavidin enrichment.

4× Laemmli buffer	
Reagent	Final concentration
Tris-HCl pH 6.8	200 mM
Ethylenediaminetetraacetic acid (EDTA)	150 mM
Dithiothreitol (DTT)	400 mM
SDS	8% (wt/vol)
Glycerol	40% (vol/vol)
ddH_2O	
Prepare freshly. DTT degrades over time.	

⚠ CRITICAL: Contact with SDS and DTT cause skin and eye irradiation. Wear protective gloves/protective clothing/eye protection/face protection. Do not inhale SDS.

10× TBS	
Reagent	Final concentration
Tris-HCl pH 7.6	500 mM
NaCl	1.5 mM
ddH_2O	
As required, dilute 1:10 to make 1× TBS. Store at 4°C–25°C for up to a year.	

CTT growth medium	
Reagent	Final concentration
Casitone	1% (wt/vol)
Tris-HCl pH 7.6	10 mM
MgSO4	8 mM
Potassium phosphate pH 7.6	1 mM
ddH_2O	
Autoclave immediately. Stable for several months at RT.	

Alternatives: Growth media have to be adjusted to the individual organism.

2.5 mM TCEP reduction buffer	
Reagent	Final concentration
Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP)	2.5 mM
Ammonium bicarbonate	100 mM
Can be aliquoted and stored at −20°C for several weeks. Avoid thaw-freeze cycles of aliquots.	

5 mM Iodoacetamide alkylation buffer	
Reagent	Final concentration
Iodoacetamide	5 mM
ddH_2O, MilliQ	
Can be aliquoted and stored at −20°C for several weeks. Avoid thaw-freeze cycles of aliquots.	

SPE Buffer 1	
Reagent	Final concentration
Trifluoroacetic acid	0.1% (vol/vol)
ddH_2O, HPLC grade	

SPE Buffer 2	
Reagent	Final concentration
Acetonitrile	50% (vol/vol)
Trifluoroacetic acid	0.1% (vol/vol)
ddH_2O, HPLC grade	

LC buffer A	
Reagent	Final concentration
Formic acid, LC-MS grade	0.15% (vol/vol)
ddH_2O, LC-MS grade	

LC buffer B	
Reagent	Final concentration
Acetonitrile, LC-MS grade	50% (vol/vol)
Formic acid, LC-MS grade	0.15% (vol/vol)
ddH_2O, LC-MS grade	

- 100 mM biotin stock solution: add 122 mg biotin in a total of 5 mL 1 M Tris pH 7.6 and titrate with KOH until biotin is fully dissolved.

Store in aliquots at −20°C for up to a year.

- 500 mM vanillate stock solution: add 840.7 mg vanillate to a total of 10 mL ddH_2O and titrate with KOH until vanillate is fully dissolved.

Store at 4°C for up to a year.

- Transfer-buffer: Add 36.3 *g* Tris and 22.5 *g* glycine; fill to 1 L with ddH_2O. Do not adjust the pH.

Stable for several months at RT.

- TBST buffer: Add 0.5 mL Tween 20 to 1 L of TBS.

Stable for several months at RT.

- 5% (wt/vol) non-fat milk in TBST: Dissolve 25 *g* of milk powder in 500 mL of 1× TBST.

Stable for several days at 4°C.

Alternatives: Concentration of milk powder may be adjusted to avoid background in western blots.

- 3% (wt/vol) Bovine serum albumin (BSA) in TBST: Dissolve 15 *g* of BSA in 500 mL of TBST.

Stable for several days at 4°C.

Alternatives: Concentration of BSA may be adjusted to avoid background in western blots.

- Kanamycin stock solution: add 500 mg kanamycin sulfate in 10 mL ddH_2O.

Store in aliquots at −20°C.

⚠ CRITICAL: Kanamycin is harmful. Wear protective gloves/protective clothing/eye protection/face protection.

- Gentamycin stock solution: add 100 mg gentamycin sulfate in 10 mL ddH_2O.

Store in aliquots at −20°C.

⚠ CRITICAL: Gentamycin is harmful. Wear protective gloves/protective clothing/eye protection/face protection.

- CTT supplemented with kanamycin and/or gentamycin: Add 1 µL per mL of the gentamycin or kanamycin stock solution to the CTT medium.

Prepare freshly.

STEP-BY-STEP METHOD DETAILS

Determine the concentrations of vanillate, biotin and labeling time for proximity labeling experiments using western blot

Timing: 1–2 weeks

In this step, the amount of inducer used to express the control protein (sfGFP-mTurbo-FLAG) is titrated to reach a level similar to that of the bait protein. Additionally, the amount of biotin and the labeling time is adjusted.

1. To adjust the accumulation of bait and control protein to similar levels, whole cell lysates of cells, grown in the presence of different concentrations of inducer (in our case vanillate), are prepared and analyzed by western blot.

a. Prepare culture of strains synthesizing the bait protein or the control protein.
   i. Resuspend a single colony per strain in 5 mL CTT liquid medium and incubate cultures with shaking for 8 h at 32°C in the dark.

Note: Incubation times should be adjusted to the doubling time of the bacterium used. *M. xanthus* cells growing in suspension culture have a doubling time of 5–6 h. Also *M. xanthus* cells are light-sensitive and therefore incubated in the dark. These parameters can be adjusted to suit other bacteria.

   ii. Dilute the cultures to an OD_{550nm} = 0.1 in 10 mL CTT. For the strain expressing the control protein, inoculate 5 cultures with an OD_{550nm} = 0.1.

b. To induce synthesis of the control protein from the vanillate inducible promoter, add vanillate to the five cultures to final concentrations of 0, 5, 10, 50, and 100 μM, and incubate the cultures with shaking for maximal 3 generations (≈18 h) at 32°C in the dark to make sure that cells remain in the exponential growth phase.

c. Take samples for SDS-PAGE by normalizing cultures to an OD_{550nm} = 14.
   i. Harvest cells by centrifugation at 4,500 *g* for 10 min at RT and resuspend to an OD_{550nm} = 14 in 1× Laemmli buffer.
   ii. Heat samples at 95°C for 10 min with shaking at 1,000 rpm.
   iii. Briefly centrifuge the samples to sediment condensation in the lid.

Pause point: Samples can be stored at −20°C for weeks.

d. Load 15 μL of each sample on an Any kD Mini-PROTEAN TGX Precast Protein Gel and run the gels at 150 V for 45 min in an electrophoresis cell.
e. Transfer the gel to 0.2 μm nitrocellulose membrane using the 7 min TurboTransfer program using the TransBlot TurboTransfer System.
f. Block the membrane with 5% milk for 1 h at RT.
g. Incubate the membrane with α-FLAG primary antibody (1:2,000 in 1% milk TBS) over night at −4°C.
h. Wash the membrane three times with 1× TBST for 5 min each.
i. Incubate the membrane with α-rabbit secondary antibody (1:15,000 in 1% milk TBS) for 1 h at −4°C.
j. Wash the membrane three times with 1× TBST for 5 min each.
k. Develop the blot using the luminescent image analyzer LAS-4000 after applying the Luminata Western HRP Substrate.
l. Strip the membrane by incubating it in 15 mL stripping-buffer for 15 min at RT.
m. Wash the membrane three times with 1× TBST for 5 min each.
n. Block the membrane with 5% milk for 1 h at RT.
o. Wash the membrane three times with 1× TBST for 5 min each.
p. Incubate the membrane with Streptavidin-HRP (1:4,000 in 3% BSA-TBS) over night at 4°C.
q. Wash the membrane three times with 1× TBST for 5 min each.
r. Develop the blot using a luminescent image analyzer after applying the Luminata Western HRP Substrate.

Note: Using western blot, we determined that 5–10 μM vanillate are sufficient to reach the same level of accumulation of the bait and control proteins (Figure 1A). Additionally, we observed that the sfGFP-mTurbo-FLAG fusion is degraded when increasing the expression (50 and 100 μM vanillate).

2. To optimize the amount of biotin added to the cells for the labeling reaction, whole cell lysates of cells grown with different biotin concentrations for 4 h are prepared and analyzed by western blot.

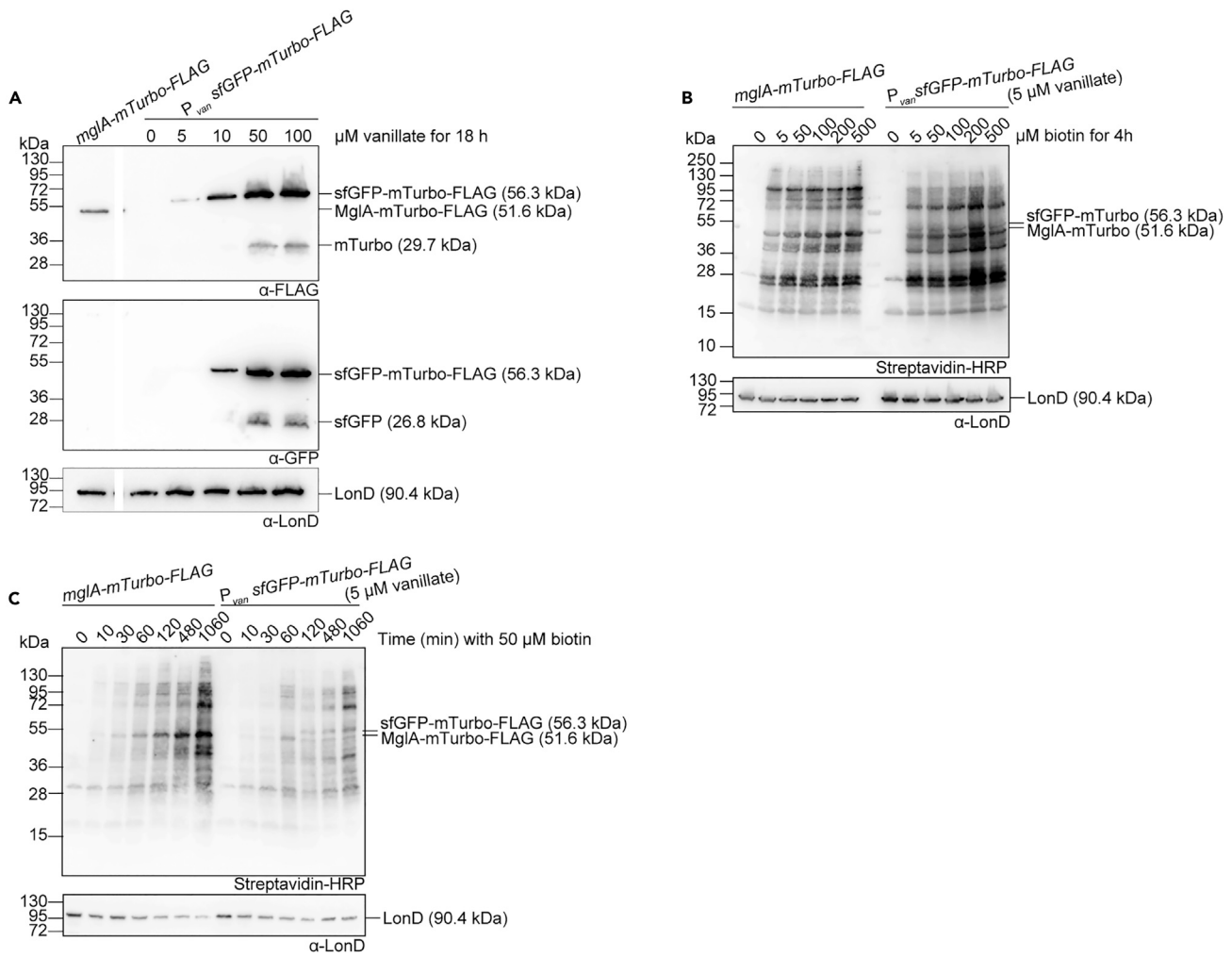


Figure 1. Optimization of vanillate concentration, biotin concentration and labeling time

(A–C) Protein from the same amount of cells was loaded in each lane and separated by SDS-PAGE. Blots were probed with the indicated antibodies or Streptavidin-HRP. Before probing with a different antibody, blots were treated with stripping-buffer. LonD was used as a loading control. In (A), gap indicates lanes removed for presentation purposes.

a. Prepare culture of strains synthesizing the bait protein or the control protein.
   i. Resuspend a single colony per strain in 5 mL CTT liquid medium and incubate cultures with shaking for 8 h at 32°C in the dark.
   ii. Dilute cultures to an OD_{550nm} = 0.1 in 10 mL CTT. For the strain expressing the control protein, inoculate 5 cultures with an OD_{550nm} = 0.1.
b. Induce synthesis of the control protein using the experimentally determined vanillate concentration and incubate culture with shaking for 18 h at 32°C (see comment in Step 1b about incubation).
c. Induce labelling by adding biotin to 0, 5, 25, 50, 100, 200, and 500 µM final concentration to the cultures. Incubate with shaking for 4 h at 32°C in the dark.
d. Take samples for SDS-PAGE by normalizing cultures to an OD_{550nm} = 14.
   i. Harvest cells by centrifugation at 4,500 *g* for 10 min at RT and resuspend to an OD_{550nm} = 14 in 1× Laemmli buffer.
   ii. Heat samples at 95°C for 10 min with shaking at 1,000 rpm.
   iii. Briefly centrifuge the samples to sediment condensation in the lid.

e. Load 15 μL of each sample on an Any kD Mini-PROTEAN TGX Precast Protein Gel and run the gels at 150 V for 45 min in an electrophoresis cell.
f. Transfer the gel to 0.2 μm nitrocellulose membrane using the 7 min TurboTransfer program using the TransBlot TurboTransfer System.
g. Block the membrane with 5% milk for 1 h at RT.
h. Wash the membrane three times with 1× TBST for 5 min each.
i. Incubate the membrane with Streptavidin-HRP (1:4,000 in 3% BSA-TBS) over night at 4°C.
j. Wash the membrane three times with 1× TBST for 5 min each.
k. Develop the blot using a luminescent image analyzer after applying the Luminata Western HRP Substrate.

Note: Generally, concentrations between 50 and 100 μM are already saturating the labeling reaction (Figure 1B). Ideally, the concentration is chosen as low as possible to reduce the amount of free biotin in the lysate.

3. The labelling time after addition of biotin should be optimized to ensure labeling level and specificity. To optimize the labeling time, whole cell lysates at different time points after addition of biotin to a fixed concentration are prepared and analyzed by western blot. For the test experiments, we used 50 μM biotin to induce the labeling reaction because this concentration yielded robust and strong labeling as determined in step 2.
   a. Prepare culture of strains accumulating the bait protein and the control. Because samples will be taken from the same culture at different time points the culture is started with a larger volume.
      i. Resuspend a single colony per strain in 5 mL CTT liquid medium and incubate cultures with shaking for 18 h at 32°C in the dark (see comment in Step 1b about incubation).
      ii. Dilute cultures to an OD_{550nm} = 0.3 in 10 mL CTT and incubate cultures with shaking for 8 h at 32°C in the dark.
      iii. Dilute cultures to an OD_{550nm} = 0.1 in 50 mL CTT.
      iv. Add vanillate to a final concentration as determined in step 1 to induce synthesis of the control (sfGFP-mTurbo-FLAG) and incubate culture with shaking for 18 h at 32°C in the dark (see comment in Step 1b about incubation).
   b. Induce labelling by adding 50 μM biotin to the cultures.
   c. Incubate the cultures with shaking for 0 min, 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, and 24 h at 32°C in the dark.
   d. Take samples for SDS-PAGE by normalizing cultures to an OD_{550nm} = 14.
      i. Harvest cells by centrifugation at 4,500 *g* for 10 min at RT and resuspend to an OD_{550nm} = 14 in 1× Laemmli buffer.
      ii. Heat samples at 95°C for 10 min with shaking at 1,000 rpm.
      iii. Briefly centrifuge the samples to sediment condensation in the lid.
   e. Load 15 μL of each sample on an Any kD Mini-PROTEAN TGX Precast Protein Gel and run the gels at 150 V for 45 min in an electrophoresis cell.
   f. Transfer the gel to 0.2 μm nitrocellulose membrane using the 7 min TurboTransfer program using the TransBlot TurboTransfer System.
   g. Block the membrane with 5% milk for 1 h at RT.
   h. Wash the membrane three times with 1× TBST for 5 min each.
   i. Incubate the membrane with Streptavidin-HRP (1:4,000 in 3% BSA-TBS) over night at 4°C.
   j. Wash the membrane three times with 1× TBST for 5 min each.
   k. Develop the blot using a luminescent image analyzer after applying the Luminata Western HRP Substrate.

Note: Labeling times likely depend on the organism and protein used and should be optimized accordingly. We determined the optimal labeling time to be 4 h, based on the observation that labeling with proteins saturated between 2 h and 8 h (Figure 1C).

Preparation and labeling of cells grown on a surface or in suspension culture

Timing: 52 h

After optimizing the vanillate and biotin concentration, as well as the labeling time, biotinylated proteins are enriched using Strep beads. To compare cells grown on a surface with cells grown in suspension culture, vanillate was added to the control cells in suspension culture and in 150 mm Petri dishes. Each strain was analyzed in biological triplicates to verify reproducibility.

4. Induction of labeling in cells in suspension culture.
   a. Resuspend single colonies in 10 mL CTT liquid medium and incubate cultures with shaking for 8 h at 32°C in the dark.
   b. Dilute cultures to an OD_{550nm} = 0.1 in 50 mL CTT, induce synthesis of the control protein by adding vanillate and incubate cultures with shaking for 18 h at 32°C in the dark (see comment in Step 1b about incubation).

   Note: Adjust vanillate concentration to the concentration determined in step 1. For the presented experiment we used 5 μM.

   c. Add biotin to each suspension culture and incubate the cells for 4 h.

   Note: Use the biotin concentration determined in step 2 and adjust the time determined in step 3, for these experiments we used 50 μM biotin.

5. Labeling in cells grown on a surface.
   a. Prepare suspension cultures of strains accumulating the bait protein and the control in biological triplicates.
      i. Resuspend a single colony per replicate in 5 mL CTT liquid medium and incubate cultures with shaking for 8 h at 32°C in the dark.
      ii. Dilute cultures to an OD_{550nm} = 0.1 in 20 mL CTT and incubate cultures with shaking for 18 h at 32°C in the dark (see comment in Step 1b about incubation).
   b. Dilute each culture in 40 mL CTT to OD_{550nm} = 0.05 in 40 mL, transfer the cell suspension to a Petri dish (Ø = 150 mm) and incubate for 8 h at 32°C in the dark.

   Note: The cells sediment at the plastic bottom of the Petri dish while being overlaid by the growth medium. Vanillate and biotin are added directly to the growth medium.

   c. To induce synthesis of the control protein from the vanillate inducible promoter, add vanillate to the growth medium covering the cells in the Petri dishes with the corresponding strains and continue for 18 h at 32°C in the dark (see comment in Step 1b about incubation).

   Note: Adjust the vanillate concentration to the concentration determined in step 1. For the presented experiment we used 5 μM.

   d. To induce the labeling reaction, add biotin to the growth medium covering the cells for 4 h at 32°C in the dark.

   Note: Adjust the biotin concentration to the concentration determined in step 2 and adjust the time determined in step 3, for the presented experiment we used 50 μM biotin.

   ⚠ CRITICAL: Do not agitate Petri dishes as agitation will lead to detachment of cells from the bottom of the Petri dish.

e. After labelling, scrape cells off the surface using a cell scraper and combine cells of the 3 Petri dishes of one replicate.

Enrichment of biotinylated proteins and on-bead digest

Timing: 20 h

After *in vivo* labeling cells are lysed and biotinylated proteins enriched using Strep beads. Subsequently, biotinylated proteins are eluted and digested using an on-bead digest procedure.

6. Enrichment of biotinylated proteins using Strep beads.
   a. Harvest cells by centrifugation at 8,000 *g* for 10 min at RT.
   b. Wash the cell pellet by resuspending in 10 mL TPM buffer.
   c. Harvest cells at 8,000 *g* for 10 min at 4°C and discard the supernatant.
   d. Repeat steps b and c three times.

   Note: Extensive washing ensures that free biotin, which could interfere with the enrichment of biotinylated proteins, is removed efficiently.

   e. Resuspend the cell pellet in 600 µL RIPA buffer supplemented with cOmplete protease inhibitor (Roche).
   f. Sonicate for 30 s with a Hielscher UP200st set to amplitude = 50% and pulse = 50%.
   g. Pellet cell debris at 8,000 *g* for 10 min 4°C. In the meantime, start equilibrating the desalting columns.
   h. Equilibrate G25-Desalting columns.

   Note: Desalting columns are used to remove any free biotin left in the lysate. Instead of a spin protocol, desalting columns can also be used with a gravity protocol for more efficient removal of free biotin.

      i. Remove the top and bottom seal of the column and remove the filter with forceps.
      ii. Load the desalting column onto a 15 mL Falcon tube using an appropriate adapter.
      iii. Load 2–3 mL of RIPA buffer on the desalting column and let the buffer drain by gravity. Repeat this step.
      iv. Load 2–3 mL of RIPA buffer on the desalting column and empty the desalting column by centrifugation at 1,000 *g* for 2 min.
   i. Transfer 500 µL of the cleared lysate on a desalting column and let the lysate enter the column bed.
   j. Transfer the desalting column into a new falcon tube and collect the cleared lysate by centrifugation at 1,000 *g* for 2 min.
   k. Measure the protein concentration in all samples.
   l. Dilute 10 µL of the desalted lysate in 90 µL ddH_2O.
   m. In a 96-well plate: Mix 10 µL ddH_2O, and 10 µL of BSA standards with 190 µL 660 nm Protein Assay. In triplicates: Mix 10 µL of the diluted lysates with 190 µL 660 nm Protein Assay.
   n. Mix all wells by shaking and measure the absorbance at 660 nm in an appropriate plate reader (i.e., Tecan M200).

   Note: Dilute samples and use BSA standards according to the linear range of the assay used to measure the protein concentration.

   o. Dilute all lysates to the same protein concentration.

   Note: We use 2 mg/mL in our experiments.

p. Equilibrate Strep beads.
   i. Resuspend 40 μL of Strep beads per sample in 1 mL RIPA buffer.
   ii. Separate beads using a magnetic rack.
   iii. Repeat wash.
   iv. Resuspend beads in 500 μL RIPA.
q. Mix 500 μL desalted lysate and 500 μL equilibrated beads in a 2 mL Eppendorf tube (corresponding to a protein concentration of 1 mg/mL during the binding step) and incubate for 1 h on a rotary shaker at 4°C.

Note: In our hands, 2 mL tubes allow easier separation of magnetic beads.

r. Separate the Strep beads from the lysate using a magnetic rack.
s. Wash the beads twice with 1 mL RIPA buffer.
t. Wash the beads twice with 1 mL 1 M KCl.
u. Wash the beads three times with 1 mL 50 mM Tris-HCl pH 7.6.

Note: As on-bead digest is performed by directly adding the endopeptidase trypsin to proteins enriched on Strep beads, all detergents and protease inhibitors have to be removed prior to adding the protease.

v. Wash the beads with 1 mL 100 mM Ammonium bicarbonate (ABC).
w. Resuspend beads in 100 μL 100 mM ABC.

Pause point: Remove ABC and snap-freeze the dry beads in liquid nitrogen. Beads can be stored at −80°C until proceeding with the on-bead digest.

⚠ CRITICAL: Liquid nitrogen can cause severe burns when in contact with the skin/ eye. Wear appropriate protection.

7. Perform on-bead digest of enriched proteins.
   a. Add 200 μL on-bead digestion buffer (1 μg trypsin in 100 mM ABC) to the beads, vortex and incubate for 30 min on a thermomixer at 30 °C at 1,200 rpm. Make sure the beads are always fully resuspended.
   b. Separate, collect supernatant and transfer into new Eppendorf tube.
   c. Add 100 μL TCEP reduction buffer to the beads, vortex and separate beads.
   d. Collect supernatant and add to trypsin containing sample.
   e. Let reaction proceed at 30°C overnight.
   f. After tryptic digest, add 5 μL alkylation buffer, vortex and incubate for 30 min in the dark.

Pause point: Proceed with solid phase extraction or freeze samples at −20°C.

Solid phase extraction of tryptic peptides

Timing: 3 h

In this step the peptide samples are desalted and prepared for LC-MS injection.

8. Acidify samples by adding 50 μL of 5% trifluoroacetic acid (TFA).
9. Condition C18-columns with 200 μl Acetonitrile and spin at 8 *g* on a table top centrifuge for 30 s.
10. Equilibrate columns with 400 μL SPE Buffer 1, centrifuge at 15 *g* for 30 s at RT.
11. Load sample and spin at 8 *g* for 1 min at RT.

Note: Depending on sample and cartridge dimensions, the centrifugation time, speed and buffer volumes may be adopted. The SPE columns should never run completely dry. Repeatedly discard the centrifugation flow through.

12. Wash loaded peptide sample by adding 400 µL Buffer 1, centrifuge at 15 *g* for 30 s at RT.
13. Transfer the SPE column with bound peptides to a new Eppendorf tube. Add 400 µL SPE Buffer 2 and centrifuge 15 *g* for 30 s at RT.
14. Concentrate eluted peptide mixture under vacuum to dryness. Drying process in a SpeedVac takes around 2 h.
15. Reconstitute peptides in 50 µL Buffer 1, vortex and transfer sample into LC vial and proceed with LC-MS analysis. Otherwise, peptides can be stored dry at −20°C.

Pause point: Peptide sample can be stored dry for several months.

LC-MS analysis

Timing: 75 min per sample

Here we describe an example for an LC-MS acquisition method suited for mTurbo affinity purification experiments. This individually established routine includes an Ultimate 3000 RSLC system connected to an Exploris 480 Orbitrap mass spectrometer using a data dependent acquisition (DDA) MS strategy for label-free protein quantification.

Note: With the variety of possible LC-MS/MS analytical set ups including column origin, important parameters can vary substantially, depending on the installed HPLC and MS system.

16. Load 5 µL peptide material onto the Pre-column with 10 µL/min flow LC-Buffer A (0.15% Formic acid).
17. For peptide separation use a self-packed C18 column with 42 cm length and 75 µm ID × 360 µm OD column heated at 60°C using an in-house designed column heater.
18. Perform peptide separation at constant flow rate of 300 nL/min using a 40 min gradient from 6%–35% LC buffer B (99.85% Acetonitrile/0.15% Formic acid) on a 42 cm self-packed analytical.

Note: The implementation of a column heater will reduce back pressure and allow the use of longer column for better peptide separation. If no column heater is used, shorter columns are used to prevent LC overpressure.

19. Eluting peptides are ionized with a spray voltage of 2.3 kV in positive mode.
20. Set the capillary temperature to 300°C.
21. Acquire MS1 survey scan (scan range m/z 350–1,650) in profile mode with following parameters:
    a. Set Orbitrap resolution to 60,000.
    b. AGC target setting to 300% ($3e^6$) and max fill time to 25 ms.
    c. RF lens 40%
22. Acquire MS/MS fragment spectra in data dependent acquisition (DDA) mode with following parameters:
    a. Quadrupole isolation window m/z 1.5.
    b. For fragmentation include ions with charge states 2–6.
    c. Set Orbitrap resolution to 15,000.
    d. AGC target setting to 200% ($2e^5$) and max fill time set to "Auto".
    e. HCD normalized collision energy 27.
    f. Dynamic exclusion 20 s.
    g. Set cycle time for MS/MS sequencing attempts to 1 s.

Optional: Next to DDA other MS acquisition strategies like data independent acquisition (DIA) are possible options for protein label-free quantification. The choice of the MS acquisition strategy is based on several factors including sample complexity and observed data robustness.

Protein label-free quantification using MaxQuant

Timing: 4 h

The MS raw data is analyzed with MaxQuant for protein label-free quantification (LFQ). MaxQuant is a widely used, freely available analysis software with several implemented strategies including label-free, isotopic and isobaric protein quantification.

23. Download MaxQuant (https://www.maxquant.org/) and install software on an appropriate PC.
24. Download *Myxococcus xanthus* protein sequence database in FASTA format via UniProt (https://www.uniprot.org/).
25. Load raw files and "set experiment" according to experimental set up.
26. Process MaxQuant with "Label-free quantification" and "min. ratio count" set to "1". For modifications set carbamidomethyl on (C) as fixed; oxidation on (M) and deamidation on (DQ) as variable. Activate "Require MS/MS for LFQ comparisons" to include only peptides identified by defined identification FDRs.
27. With few exceptions MaxQuant is best performed in standard settings.
28. Locate the MaxQuant output ".txt" folder and use the "LFQ intensities" in the "proteinGroups.txt" output for further analysis.

Note: The MaxQuant output tables offer detailed information on identified peptides and proteins helpful for further monitoring and troubleshooting. In order to evaluate comparability across samples and to avoid label-free quantification biases make sure homogenous distribution of number unique peptides and intensities found in the "proteinGroups.txt" table.

EXPECTED OUTCOMES

The presented protocol allows the identification of the protein interactome of the key motility regulator MglA in live *M. xanthus* cells under two different conditions using mTurbo-based proximity labeling. First, key parameters (i.e., protein expression, biotin concentration and labeling time) were optimized. Then, the optimized procedure was applied to study the protein interactome of MglA in two different cell states.

After conducting the proximity labeling, LC-MS/MS analysis and label-free protein quantification, the significance and degree of protein enrichment versus control strains are calculated using Perseus[20] and visualized in volcano plots. Using defined enrichment criteria (log2ratio ≥ 2; p-value ≤ 0.03), several proteins were identified that are indicative of the general success of the proximity labeling experiment:

First, the MglA-mTurbo-FLAG bait was enriched, representing an important experimental quality control. It is noteworthy that MglA did not show strong enrichment. This is likely caused by the general high MglA background abundance, the small size of MglA (22 kDa) and potentially reduced tryptic cleavage sites due to biotinylation (see limitations). Second, inspection of the data revealed the enrichment of proteins that have previously been shown to interact with MglA and include direct protein interactors and/or proteins functionally linked to motility. These proteins are highlighted in Figure 2. Specifically, among proteins known to interact with MglA, we identified the GAP proteins MglB and RomY,[13,14,16] the gliding motility protein AglZ[8,11] and GltJ[21] and the regulators of T4aP-dependent motility FrzS and SgmX,[7,9,10] demonstrating the feasibility of the mTurbo

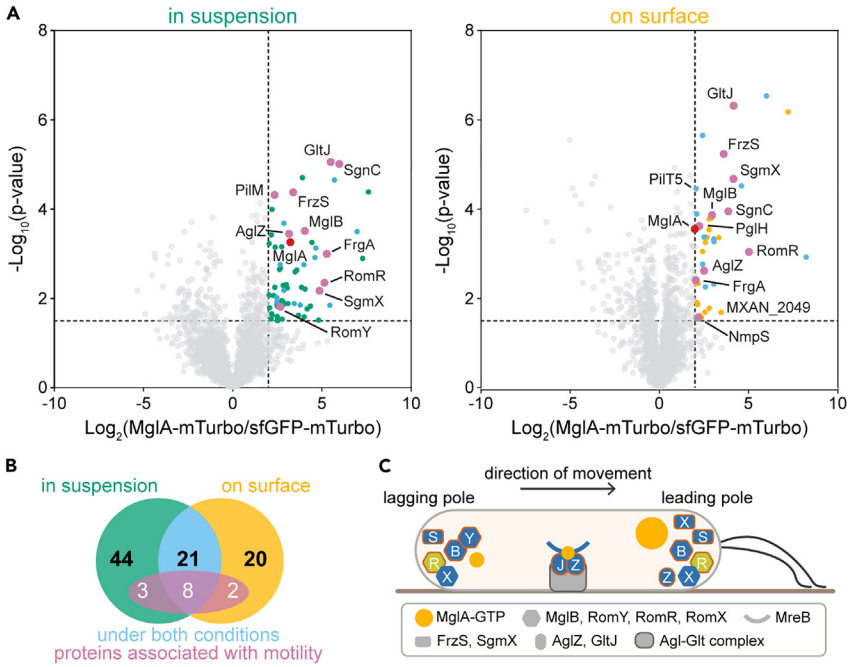


Figure 2. Identification of known and new potential interaction partners of MglA

(A) Volcano plots representing the results from proximity labeling experiments with cells expressing MglA-mTurbo-FLAG during surface growth and growth in suspension culture. Plots show the comparison of MglA-mTurbo-FLAG and sfGFP-mTurbo-FLAG from suspension cultures (left) and during surface growth (right). The X-axis represents the log2ratio of group mean intensities from three experiments. The Y-axis represents the statistical significance of the corresponding targets. Dashed lines represent the threshold (log2ratio>2; p-value<0.03). Points represent identified proteins, MglA is shown in red, remaining proteins are indicated using the same color code as in (B). Known motility proteins or regulatory proteins are labeled with their names.

(B) Venn diagram showing the number of proteins enriched exclusively in suspension cultures (green), exclusively during surface growth (yellow) and under both conditions (blue). Known motility proteins or regulatory proteins in these three categories are shown (pink). Colors correspond to colored points in the volcano plots in (A).

(C) Schematic cell with the localization of known interaction partners of MglA. Proteins corresponding to their shapes described in the legend below. Letter within shape represents the last letter of a protein. Blue indicates known direct interaction partners of MglA. Green indicates protein that has not been shown to directly interact with MglA. Orange outlines mark proteins identified in the proximity labeling experiments.

proximity labeling protocol. Of note, while most of these interactions were verified using *in vitro* methods and purified proteins, proximity labeling was able verify these interactions *in vivo*. Third, we identified RomR, which is part of the MglA GEF but likely does not interact directly with MglA (Szadkowski 2019). Fourth, we identified other proteins with a verified function in motility including PilM, which is essential for T4aP-dependent motility.[22] Fifth, we identified FrgA,[23] SgnC,[24] NmpS[25] and PglH,[26] all of which have been implicated in motility or its regulation. Because PilM, GltJ, SgnC and PglH were identified as potential interaction partners of MglA, they are interesting candidates to further extend our understanding of how MglA stimulates the two motility systems.

Because external conditions, such as surface contact, can influence the subcellular localization of proteins in bacteria on short time scales, e.g., in *Pseudomonas aeruginosa* proteins that promote surface colonization localize within seconds after surface contact,[27] we conducted proximity labeling under two different conditions (growth on surface and suspension culture). Although many of the potential interactions were detected independently of the two conditions), we identified distinct candidates for the two conditions. For example, PglH and the DnaJ/TPR domain protein MXAN_2049, which is encoded directly next to *pglH*, and the PilT5 protein, which is a paralog of the T4aP retraction ATPase PilT, were only enriched when cells were grown on a surface, while PilM was only enriched in cells grown in suspension culture. These observations suggest that the interaction network

of MglA changes upon surface contact. Thus, conducting the proximity labelling under different environmental conditions can help to identify conditional interactions and generate snapshots of condition-dependent protein interactions.

LIMITATIONS

Despite the potential of proximity labeling to uncover protein interactomes, some limitations are associated with this approach. Inherently, the method does not allow to distinguish whether a significantly enriched protein directly interacts with the bait, or if the interaction is indirect. Alternative methods (i.e., pull-down assay, FRET-microscopy and co-immunoprecipitation) have to be used to further distinguish between direct and indirect interaction.

Enrichment and identification of small proteins or membrane proteins can be challenging due to a lack of (surface exposed) Lys residues. Furthermore, biotinylated Lys residues provide a poor substrate for tryptic digestion and, consequently, are more difficult to identify due to a higher degree of miscleaved products.

For transmembrane proteins the topology should be considered, since the periplasm lacks ATP, the biotinylation reaction does not work in that compartment. This additionally limits TurboID based proximity labeling to the cytoplasm or to cytoplasmic parts of IM proteins. As an alternative, APEX2-based proximity labeling has been reported for periplasmic proteins.[28]

In addition, the proximity labeling reaction is mainly regulated by the availability of biotin and temperature and therefore is difficult to control on short timescales. UV light inducible systems like the recently introduced LOV-TurboID[29] may provide improved temporal control of the labeling reaction.

TROUBLESHOOTING

Problem 1

The mTurbo-fusion is degraded (Step 1).

Potential solution

Changing the sequence or length of the linker between protein of interest and mTurbo may increase fusion stability. Also fusing mTurbo to a different terminus of the bait protein may increase stability. Alternatively, other proximity labeling tags, such as TurboID[1] or UltraID[30] could be tested for enhanced stability.

Problem 2

The mTurbo-fusion is not stable or not synthesized (Step 1).

Potential solution

Change the promoter used for the expression of the fusion protein or codon optimization also can enhance the efficiency of protein expression.

Problem 3

The mTurbo-bait protein does not bind to the Strep beads/ proportionally high amount of bait-protein remains in the flow through (Step 6).

Potential solution

This could be due to an excess of free biotin in the lysate.

- Increase the amount of washing steps (Step 6.a) or the washing volume of the culture.
- Switch from spin protocol to gravity protocol for the desalting column.
- Check and possibly increase the volume of beads used for affinity purification.

Problem 4
Varying protein recovery across samples after LC-MS analysis (Step 22).

Potential solution
Optimize the lysis condition including choice of detergent, heat exposure, disruption method (e.g., sonication, bead disruption, etc.) and enzymatic support (e.g., lysozyme).

Problem 5
Contaminated chromatograms and/or poor protein recovery after LC-MS analysis despite enrichment confirmed by western blot (Step 22).

Potential solution
Include more bead washing steps before on-bead digest or adopt optimize protein elution/digest strategy (e.g., detergent and heat elution with subsequent acetone precipitation and in-solution digest).

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Timo Glatter (timo.glatter@mpi-marburg.mpg.de).

Materials availability
Plasmids and strains are available upon request.

Data and code availability
The dataset generated during this study are available upon request.

ACKNOWLEDGMENTS
The authors thank María Pérez Burgos for construction of plasmids and strains. This work was generously funded by the Max Planck Society.

AUTHOR CONTRIBUTIONS
M.H., F.M., and T.G. performed the experiments and analyzed data. T.G. performed MS analysis. M.H., F.M., L.S.-A., and T.G. wrote the manuscript. L.S.-A. acquired funding. L.S.-A. and T.G. provided supervision.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES
1. Branon, T.C., Bosch, J.A., Sanchez, A.D., Udeshi, N.D., Svinkina, T., Carr, S.A., Feldman, J.L., Perrimon, N., and Ting, A.Y. (2018). Efficient proximity labeling in living cells and organisms with TurboID. Nat. Biotechnol. *36*, 880–887. https://doi.org/10.1038/nbt.4201.

2. Qin, W., Cho, K.F., Cavanagh, P.E., and Ting, A.Y. (2021). Deciphering molecular interactions by proximity labeling. Nat. Methods *18*, 133–143. https://doi.org/10.1038/s41592-020-01010-5.

3. Carreira, L.A.M., Szadkowski, D., Müller, F., and Søgaard-Andersen, L. (2022). Spatiotemporal regulation of switching front-rear cell polarity. Curr. Opin. Cell Biol. *76*, 102076. https://doi.org/10.1016/j.ceb.2022.102076.

4. Zhang, Y., Ducret, A., Shaevitz, J., and Mignot, T. (2012). From individual cell motility to collective behaviors: insights from a prokaryote, Myxococcus xanthus. FEMS Microbiol. Rev. *36*, 149–164. https://doi.org/10.1111/j.1574-6976.2011.00307.x.

5. Schumacher, D., and Søgaard-Andersen, L. (2017). Regulation of cell polarity in motility and cell division in Myxococcus xanthus. Annu. Rev. Microbiol. *71*, 61–78. https://doi.org/10.1146/annurev-micro-102215-095415.

6. Dinet, C., and Mignot, T. (2023). Unorthodox regulation of the MglA Ras-like GTPase controlling polarity in Myxococcus xanthus. FEBS Lett. *597*, 850–864. https://doi.org/10.1002/1873-3468.14565.

7. Bautista, S., Schmidt, V., Guiseppi, A., Mauriello, E.M.F., Attia, B., Elantak, L., Mignot, T., and Mercier, R. (2023). FrzS acts as a polar beacon to recruit SgmX, a central activator of type IV pili during Myxococcus xanthus motility. EMBO J. *42*, e111661. https://doi.org/10.15252/embj.2022111661.

8. Mauriello, E.M.F., Mouhamar, F., Nan, B., Ducret, A., Dai, D., Zusman, D.R., and Mignot, T. (2010). Bacterial motility complexes require the actin-like protein, MreB and the Ras homologue, MglA. EMBO J. *29*, 315–326. https://doi.org/10.1038/emboj.2009.356.

9. Mercier, R., Bautista, S., Delannoy, M., Gibert, M., Guiseppi, A., Herrou, J., Mauriello, E.M.F., and Mignot, T. (2020). The polar Ras-like

GTPase MglA activates type IV pilus via SgmX to enable twitching motility in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA *117*, 28366–28373. https://doi.org/10.1073/pnas.2002783117.

10. Potapova, A., Carreira, L.A.M., and Søgaard-Andersen, L. (2020). The small GTPase MglA together with the TPR domain protein SgmX stimulates type IV pili formation in M. xanthus. Proc. Natl. Acad. Sci. USA *117*, 23859–23868. https://doi.org/10.1073/pnas.2004722117.

11. Treuner-Lange, A., Macia, E., Guzzo, M., Hot, E., Faure, L.M., Jakobczak, B., Espinosa, L., Alcor, D., Ducret, A., Keilberg, D., et al. (2015). The small G-protein MglA connects to the MreB actin cytoskeleton at bacterial focal adhesions. J. Cell Biol. *210*, 243–256. https://doi.org/10.1083/jcb.201412047.

12. Carreira, L.A.M., Tostevin, F., Gerland, U., and Søgaard-Andersen, L. (2020). Protein-protein interaction network controlling establishment and maintenance of switchable cell polarity. PLoS Genet. *16*, e1008877. https://doi.org/10.1371/journal.pgen.1008877.

13. Leonardy, S., Miertzschke, M., Bulyha, I., Sperling, E., Wittinghofer, A., and Søgaard-Andersen, L. (2010). Regulation of dynamic polarity switching in bacteria by a Ras-like G-protein and its cognate GAP. EMBO J. *29*, 2276–2289. https://doi.org/10.1038/emboj.2010.114.

14. Szadkowski, D., Carreira, L.A.M., and Søgaard-Andersen, L. (2022). A bipartite, low-affinity roadblock domain-containing GAP complex regulates bacterial front-rear polarity. PLoS Genet. *18*, e1010384. https://doi.org/10.1371/journal.pgen.1010384.

15. Szadkowski, D., Harms, A., Carreira, L.A.M., Wigbers, M., Potapova, A., Wuichet, K., Keilberg, D., Gerland, U., and Søgaard-Andersen, L. (2019). Spatial control of the GTPase MglA by localized RomR-RomX GEF and MglB GAP activities enables Myxococcus xanthus motility. Nat. Microbiol. *4*, 1344–1355. https://doi.org/10.1038/s41564-019-0451-4.

16. Zhang, Y., Franco, M., Ducret, A., and Mignot, T. (2010). A bacterial Ras-like small GTP-binding protein and its cognate GAP establish a dynamic spatial polarity axis to control directed motility. PLoS Biol. *8*, e1000430. https://doi.org/10.1371/journal.pbio.1000430.

17. Kaimer, C., and Zusman, D.R. (2013). Phosphorylation-dependent localization of the response regulator FrzZ signals cell reversals in Myxococcus xanthus. Mol. Microbiol. *88*, 740–753. https://doi.org/10.1111/mmi.12219.

18. Treuner-Lange, A., Chang, Y.W., Glatter, T., Herfurth, M., Lindow, S., Chreifi, G., Jensen, G.J., and Søgaard-Andersen, L. (2020). PilY1 and minor pilins form a complex priming the type IVa pilus in Myxococcus xanthus. Nat. Commun. *11*, 5054. https://doi.org/10.1038/s41467-020-18803-z.

19. Kaiser, D. (1979). Social gliding is correlated with the presence of pili in Myxococcus xanthus. Proc. Natl. Acad. Sci. USA *76*, 5952–5956. https://doi.org/10.1073/pnas.76.11.5952.

20. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., and Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat. Methods *13*, 731–740. https://doi.org/10.1038/nmeth.3901.

21. Mignot, T., Attia, B., My, L., Castaing, J.P., Dinet, C., Le Guenno, H., Schmidt, V., Espinosa, L., Anantharaman, V., and Aravind, L. (2023). A novel molecular switch controls assembly of bacterial focal adhesions. Researchsquare. https://doi.org/10.21203/rs.3.rs-2617569/v1.

22. Friedrich, C., Bulyha, I., and Søgaard-Andersen, L. (2014). Outside-in assembly pathway of the type IV pilus system in Myxococcus xanthus. J. Bacteriol. *196*, 378–390. https://doi.org/10.1128/JB.01094-13.

23. Cho, K., Treuner-Lange, A., O'Connor, K.A., and Zusman, D.R. (2000). Developmental aggregation of Myxococcus xanthus requires frgA, an frz-related gene. J. Bacteriol. *182*, 6614–6621. https://doi.org/10.1128/JB.182.23.6614-6621.2000.

24. Youderian, P., and Hartzell, P.L. (2006). Transposon insertions of magellan-4 that impair social gliding motility in Myxococcus xanthus. Genetics *172*, 1397–1410. https://doi.org/10.1534/genetics.105.050542.

25. Bretl, D.J., Ladd, K.M., Atkinson, S.N., Müller, S., and Kirby, J.R. (2018). Suppressor mutations reveal an NtrC-like response regulator, NmpR, for modulation of type-IV pili-dependent motility in Myxococcus xanthus. PLoS Genet. *14*, e1007714. https://doi.org/10.1371/journal.pgen.1007714.

26. Yu, R., and Kaiser, D. (2007). Gliding motility and polarized slime secretion. Mol. Microbiol. *63*, 454–467. https://doi.org/10.1111/j.1365-2958.2006.05536.x.

27. Laventie, B.J., Sangermani, M., Estermann, F., Manfredi, P., Planes, R., Hug, I., Jaeger, T., Meunier, E., Broz, P., and Jenal, U. (2019). A surface-induced asymmetric program promotes tissue colonization by Pseudomonas aeruginosa. Cell Host Microbe *25*, 140–152.e6. https://doi.org/10.1016/j.chom.2018.11.008.

28. Ganapathy, U.S., Bai, L., Wei, L., Eckartt, K.A., Lett, C.M., Previti, M.L., Carrico, I.S., and Seeliger, J.C. (2018). Compartment-specific labeling of bacterial periplasmic proteins by peroxidase-mediated biotinylation. ACS Infect. Dis. *4*, 918–925. https://doi.org/10.1021/acsinfecdis.8b00044.

29. Lee, S.Y., Cheah, J.S., Zhao, B., Xu, C., Roh, H., Kim, C.K., Cho, K.F., Udeshi, N.D., Carr, S.A., and Ting, A.Y. (2023). Engineered allostery in light-regulated LOV-Turbo enables precise spatiotemporal control of proximity labeling in living cells. Nat. Methods *20*, 908–917. https://doi.org/10.1038/s41592-023-01880-5.

30. Kubitz, L., Bitsch, S., Zhao, X., Schmitt, K., Deweid, L., Roehrig, A., Barazzone, E.C., Valerius, O., Kolmar, H., and Béthune, J. (2022). Engineering of ultraID, a compact and hyperactive enzyme for proximity-dependent biotinylation in living cells. Commun. Biol. *5*, 657. https://doi.org/10.1038/s42003-022-03604-5.