

Supplementary:

Kildemoes *et al.* "Detection of human IgG antibodies against *Mycoplasma genitalium* using a recombinant MG075 antigen"

M. genitalium MG075F1 cloning, production and purification

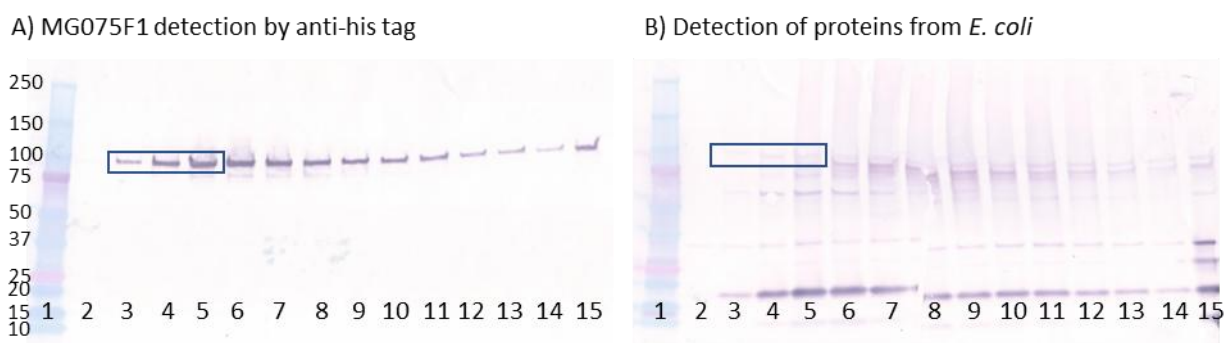
The N-terminal MG075F1 fragment was amplified using primers designed based on the *M. genitalium* G37 strain (accession no. L43967.2) for use in conjunction with the pET 102 TOPO® vector and expression system (Thermo Fisher Scientific, Invitrogen K102-01, Hvidovre, Denmark). Forward primer sequence was 5'-CACCATGAAATTAAGTACAATACTACTATC-3' (cloning tag underlined) and reverse primer 5'-AACTTCAAATAAGGATTGGAGAGTTCAAC-3'. As the usual stop codon TGA codes for tryptophan in mycoplasma species, the fragment length of 798 amino acids was determined by the first TGA occurrence in the reference sequence. DNA extracted from the Danish version of the *M. genitalium* G37 strain (4) was used as template for standard PCR amplification using the Platinum high fidelity *Taq* polymerase (Invitrogen, Thermo Fisher Scientific, Hvidovre, Denmark). PCR products were purified (Qiagen QIAquick®, Qiagen, Hilden, Germany) and cloned into the plasmid vector with C-terminal 6x His-tag and carrying ampicillin as selection marker according to manufacturer's protocol (Invitrogen K102-01, Thermo Fisher Scientific, Hvidovre, Denmark). After plasmid amplification in competent Top 10 *E. coli* and purification, the correct vector construct was confirmed by Sanger sequencing. The full amino acid sequence of the MG075F1 construct (~107kDa, isoelectric point ~6) including expressed vector components is as follows:

MGSDKI IHLTDDSFDTDLKADGAILVDFWAHWCGPCKMIAPILDEIAD EYQGKLTVAKL NIDHNPGTAPKYGIRGIPTLL
LFKNGEVAATKVGALSKGQKEFLDANLAGSGSGD LKIDPFTMKLSTITTTICLSISGAFGTTAIALPTTVALLKNHQ
QQNTEKQQNP IKDIRFGLNNVQVPNTIPLHQTVVEVTNNKAIVDYKDAPQKFFLAKSALNNKLQVEFDKFLLRGTGVINALN
ADLKEWIDQTLFIPNQSFDDL SANKLNLTLSNQSEVSLDLEFI FTNFSDKNQPLKLPFDGSSVVNANESYTSVKATLQKL
KVLTYSRADHSVGISYAIPTVSLNGKTQNDFSFNPFKSNINFAFKNVYNALNPFEAQQYLVGQKFLNQKVNADDVKNDIN
NHIETQFNVAKITATLLGKAFKQFGEHKNGQPLSLLKVL SGLNNEFKQLFN YVRPGLGDFVSDLIQSSSQSSNKKTVYQLL
FENKTTI IHLQLDLNISELNSVLPVVDILFEGINSAESLYQRIQSFKDLIVPALKADKQLKSLEAI ILAVLDNPNNTYVFDL
VYQNKSI LFNLLSDFLKNNTANTLPFLQE QFDIVNHLFANEAFD LFSNADFVEKIADLFLAKQKVQEVNNDGTSKTKIVDS
ILVATLKGVLGDQLSSITELNIIYIFENEFLNRNDSNSSVKQQTDSLKNLFSVIGDILSETNVNKITLHAVKNNELLSLV
ETASTLKIKHLNVQYKVLVDKFELKNSFIKELLNFFPDTKDITPTIKKVLFESENYKTLRKKYENEGFPGYHWAKFIVPGT
FNSAENTFYSAIDKTKSIRDLFADMLFGKSLESVNDSDSFIKINGSFTLKYHGDNLNLLPNYHSLITKNVGYQIVNVNFHI
DARLLTAELQNTVFSNPKPKVIKSPVELSKSLFEVKGELKLEGKPIPNPLLGLDSTRTGHHHHHH

Colour code: Thioredoxin, enterokinase site, MG075F1, V5 epitope, His tag, Spacer

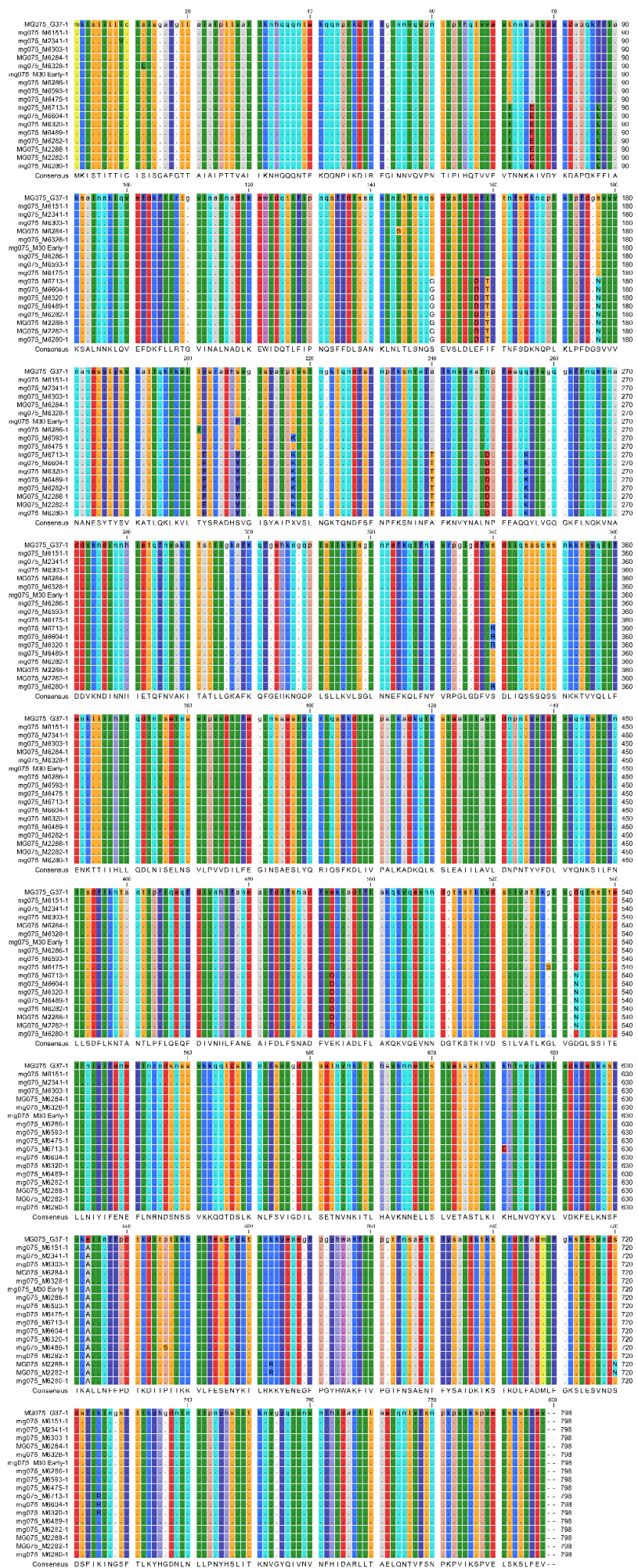
The MG075F1 plasmid was transformed into BL21-(DE3) *E. coli* by electroporation and cultured under 0,1mg/ml ampicillin pressure in LB medium (Sigma-Aldrich L3397, Merck, Søborg, Denmark). Protein fragments were produced by induction of the BL21-(DE3) MG075F1 strain with 0.5 mM IPTG (OmniPur, Sigma-Aldrich 5810, Merck, Søborg, Denmark) at OD ~0,5 culture inoculation. After ~4 hours of growth, cells were harvested by centrifugation at 2500 x g 4°C for 10 min and stored at -20°C temporarily. Successful induction of MG075F1 was confirmed by western blotting of crude boiling lysate material as described under SDS-PAGE and immunoblotting using 4-20% precast TGX-gels (BioRad, Copenhagen, Denmark; #4561091) with anti-his-AP (1:2500, Thermo Scientific, Hvidovre, Denmark, Novex R932-25) as detection antibody. Pellets were resuspended in ice-cold lysis-buffer of 400 mM NaCl, 50 mM Tris-HCL, 1% Triton X-100 (Merck, 108603) with EDTA-free protease inhibitors (Roche, Vallensbæk, Denmark, 4693159001) and transferred to tubes with 0,1-0,15mm zirconia-silicate beads (Biospec Products, Inc. Bartlesville, OK, USA; #110709101z). Mechanical lysis was done on a Tissuelyser II (Qiagen, Hilden, Germany) for 3 min at 30 Hz twice and kept on ice for five minutes in between runs. Subsequently, material was pooled again and sonicated for 3 min with 4 sec on and 3 sec off at 57% output on a Sonoplus probe sonicator (Bandelin, Berlin, Germany). After a brief sedimentation of the zirconium beads, the supernatant was then centrifuged in new tubes at 22000 x g at 4°C for 30 min. The expressed MG075F1 forms inclusion bodies, so the pellets were washed twice in the lysis

buffer as above and once without Triton X-100 and then resuspended in 6 M GuHCl, 50 mM Na₂HPO₄, 400 mM NaCl (pH 8.0). The MG075F1 in buffer was sterile filtered before it was loaded onto a HisTrap™HP nickel column (1 ml Cytiva 17524701, Vallengsbæk Strand, Denmark) on an NGC™ medium pressure liquid chromatography system (BioRad, with ChromLab version 6.0.0.35) with a fraction collector. The column was then washed in 8 M urea (Merck 108487), 100 mM Na₂HPO₄, 10 mM Tris-HCl (pH 8.0) and elution performed on a gradient by introduction of 8 M urea, 50 mM Na₂HPO₄, 500 mM imidazole (pH 8.0) and 1 ml fractions collected. Fractions with MG075F1 were identified by western blotting using 15-well precast gels (BioRad 4568096) with anti-his-AP as detection antibody. Fractions with positive anti-his signal were pooled and dialysed against 6 M urea, 20 mM Tris-HCl (pH 8.0) over a 10 kD MWCO snakeskin membrane (Thermo Scientific 88245). Anion exchange was subsequently performed on a HiTrap™Q HP column (1 ml, Merck, Cytiva 17115301) with gradient elution by introduction of 6 M urea, 20 mM Tris-HCl, 1 M NaCl (pH 8.0) and collection of fractions. Location of MG075F1 in fractions was confirmed by anti-his-AP western blotting. Presence of non-target protein was determined by Western blotting (polyclonal rabbit-anti-*E. coli* 1:1000 BioRad 4329-4906; goat-anti-rabbit-IgG-AP 1:3000 BioRad 170-6518) and standard silver-staining of gels. Fractions with high content of MG075F1 (107 kDa) and low content of non-target protein were chosen (see Suppl. Figure S1). Total protein concentrations in chosen fractions were estimated using a QUBiT protein assay (Thermo Scientific, Invitrogen Q33211) and a QUBIT fluorometer according to manufacturer's instructions. A pool of the chosen fractions had a final estimated concentration of 0.092 µg/µl protein and contained predominantly MG075F1. A total of 1 µg protein was determined as suitable for line-blotting through antigen titration in combination with incubation of known positive and negative sera and anti-his stain controls.



Suppl. Figure S1: Representative Western blots of protein fractions collected after anion exchange

Fractions loaded into lane 3, 4, and 5 (boxed) as shown on western blots for A) anti-his tag (MG075F1 107 kDa) and B) crude anti-*E. coli* proteins were chosen. These fractions had suitably low content of non-target protein with molecular weight just under the required 107kDa present in fractions from lane six and higher on B). Lane 1 contains a 10-250 kDa ladder (BioRad 161-0374) as specified on A). Lane 15 contains a pre-dialysis and anion exchange control.

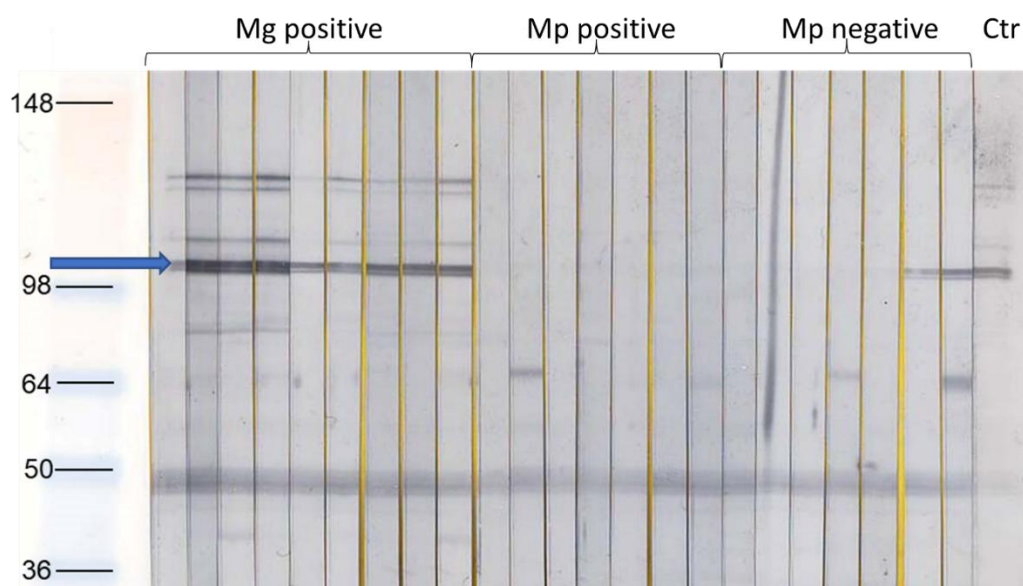


Suppl. Figure S2: MG075F1 is highly conserved between *M. genitalium* strains

The vector containing MG075F1 constructed in this paper is based on the G37 Danish isolate. Here alignment to 17 other unique *M. genitalium* MG075F1 sequences from isolates collected between 1980 and 2010 and representing five European countries, Japan and Australia are shown. These sequences as well as full genome sequence from ten other isolates with non-unique MG075F1 sequences can be extracted from the additional material included with Fookes *et al.* 2017 (4). Alignment was done using CLC Genomics Workbench ver. 20.0.

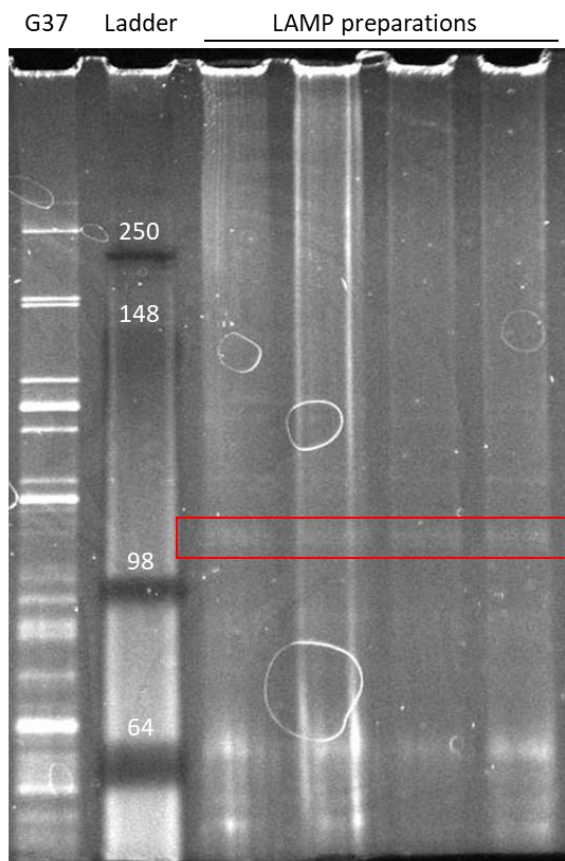
Identification of MG075 in lipid-associated membrane protein (LAMP) preparations

Lipid-associated membrane proteins (LAMPs) were prepared from the *M. genitalium* G37 type strain as previously described (1, 2) and separated on precast 4–12% Tris-glycine gradient gels (1.0 mm thick, Invitrogen, Thermo Fisher Scientific, Hvidovre, Denmark) under reducing conditions. The proteins were transferred to Invitrolon PVDF (Invitrogen, Thermo Fisher Scientific, Hvidovre, Denmark) at 20V for 2 hr, followed by incubation in Western Blocker solution (Sigma-Aldrich, Merck, Søborg, Denmark) at room temperature for at least 30 min. Immunoblotting was carried out using human sera diluted 1:200. Horseradish peroxidase (HRP)–labeled polyclonal antibodies against human IgG diluted 1:2,000 were used. Sera from *M. genitalium* NAAT positive patients reacted strongly with an approximately 110 kD antigen whereas sera from *M. pneumoniae* complement fixation test (MPT) positive patients were negative as were most sera from MPT negative children (Figure S1). In order to identify the 110 kD antigen, four LAMP preparations present in the hydrophobic phase made under the same conditions and the total cell lysate of the G37 type strain were resolved on an 8% Tris-glycine SDS-PAGE gel (1.5 mm thick, Invitrogen, Thermo Fisher Scientific, Hvidovre, Denmark) under reducing conditions. The gel was stained with SYPRO Ruby protein stain (Molecular Probes, Invitrogen, Thermo Fisher Scientific, Hvidovre, Denmark) and the bands of approximately 110 kDa from the four LAMP preparations were excised and pooled. Following trypsin digestion, the peptides were subjected to mass spectrometry (MS). MS analysis showed that the ~110 kDa band contained protein sequence matching MG075 (score 232) as identified by peptide query on the Mascot server (Matrix Science Ltd) (Figure S2B).



Suppl. Figure S3: Western blot of sera from *M. genitalium* NAAT positive patients (Mg positive) reacting with an approximately 110 kD antigen (blue arrow) whereas sera from *M. pneumoniae* complement fixation test (MPT) positive patients (Mp positive) were negative as were most sera from MPT negative children (Mp negative). On the right most side a pool of known Mg positive sera was used as positive control. The Mp positive and negative samples are from Danish children as described in the main manuscript. These Mg positive samples were from Louisiana State University, United States of America, where written informed consent was obtained from participants and the study protocol was approved by the Institutional Review Board of the Louisiana State University Health Sciences Center (3).

A) Bands selected for mass spectrometry



B) Mascot Search Results



Protein View

Match to: Y075_MYCGE Score: 232
 Uncharacterized protein MG075 OS=Mycoplasma genitalium GN=MG075 PE=4 SV=1
 Found in search of C:\calibur data file\MS09-0402-ML\MS09-0402-ML_T.RAW

Nominal mass (M): 116353; Calculated pI value: 7.98
 NCBI BLAST search of Y075_MYCGE against nr
[Unformatted sequence string for pasting into other applications](#)

[Taxonomy: Mycoplasma genitalium](#)

Variable modifications: Oxidation (M)
 Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
 Sequence Coverage: 16%

Matched peptides shown in **Bold Red**

1 MKLSTITTC LSISGAFGTT AIALPTTVAL LKNHQQQNTK **KQONPIKDIR**
 51 FGLNNVQVPN TIPLHQTVE VTNNKAIVDY **KDAPQKFLEA** KSALNNKLQV
 101 EFDKFLRTG VINALNADLK EMDQTLFIP NQSFDFLSAN KLNLTLSNQS
 151 EVSLDLEFIF TNFSDKNQPL KLPFDGSSVVNANESYTSV KATLQK**LKVL**
 201 **TYSRADHSVG** ISYAIPTVSL NGK**TQND****SF** **NPFK**SNINFAFKNVYNALNP
 251 FEAQQYLVGQ GKFLNQKVNADVKNDINNH IETQFNVAK**TATLLGKAFK**
 301 QFGEHKNQQLSLL**KVL****SGL** **NNEFK**QLFNY VRPGLGDFVS DLIQSSQSS
 351 NIKITVYQLLF ENKTTIHLL QDLNISELNS VLPVVDILFE GINSAESLYQ
 401 RIQSFKDLIV PALKADKQLK SLEAILAVL DNPNTYVFDL VYQNKSLFN
 451 LLSDFLKNTA NTLPLQEQF DIVNIHLFANE AIFDLFSNAD FVE**KIADLFL**
 501 **AKQK**QVEVNN DGTGKST**KVD** **SILVATL****KGL** VGDQLSSITE LLNIYIFENE
 551 FLNRNDSNSS VKKQQTDSLK NLFVIGDIL SETNVN**KITL** **HAVK**NNELLS
 601 LVETASTLK**I** **KHLNVQYKVL** VDKFELKNSF IKELLNFFPD TKDIT**PTIKK**
 651 **VLFESENYKT** **L****R****K**KYENEGF PGYHWAKFIV PGTFNSAENT FYSAIDKTKS
 701 IRDLFADMLF GK**SLESVND****S****DSFIKING****SF** **TLKY**HGDNLN LLPNYHSLIT
 751 KNVGYQIVNV NFHIDARLLT AELQNTVFSN PKPMKSPVE LSKSLFEVWK
 801 TIFENSVNQI LK**KEYTFKDN** **LKFFPF**KADG SSR**LEFDL****SK** **PDQR**VIPFAF
 851 VDGYYQQLK**K** **ELIPNKET****K** EANSSPVLLK YDAVKRNDRO YRPNHHDDL
 901 RNYPSLSKQL ELILNLGDKL KANNDIDDT VVNALQYKTS FKSTLKVNLSL
 951 GIPINLFFFT LWLFNLEIP IDGSLTTSV NVVFPYSLYD TSSNEFTRIV
 1001 DRLNFTDTNF YLKDAFPNFWFVG

Suppl. Figure S4: Confirmation of presence of MG075 in LAMP preparations by gel electrophoresis and mass spectrometry

A) SYPRO Ruby protein stained SDS-PAGE gel with the following loaded (left to right): Total strain G37 protein preparation, reference protein ladder (band size in kDa given in white just above bands), four LAMP preparations. Box and arrow indicate the bands cut out and analysed by mass spectrometry. B) Mascot server search output confirming presence of MG075 in the bands cut from the gel. Note that A) is best visualized if opened on devices with (O)LED or other high contrast displays.

References

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2. Jurstrand M, Jensen JS, Magnuson A, Kamwendo F, Fredlund H. 2007. A serological study of the role of Mycoplasma genitalium in pelvic inflammatory disease and ectopic pregnancy. Sex Transm Infect 83:319-23.
3. Ma L, Jensen JS, Mancuso M, Hamasuna R, Jia Q, McGowin CL, Martin DH. 2012. Variability of trinucleotide tandem repeats in the MgPa operon and its repetitive chromosomal elements in Mycoplasma genitalium. Journal of Medical Microbiology 61:191-197.
4. Fookes MC, Hadfield J, Harris S, Parmar S, Unemo M, Jensen JS, Thomson NR. 2017. Mycoplasma genitalium: whole genome sequence analysis, recombination and population structure. BMC Genomics 18:993.