TraG encoded by the pIP501 type IV secretion system is a two domain peptidoglycan degrading enzyme essential for conjugative transfer

Karsten Arends[1]†, Ertugrul-Kaan Celik[1,2]*, Ines Probst[3], Nikolaus Goessweiner-Mohr[2], Christian Fercher[2], Lukas Grumet[2], Cem Soellue[1,2], Mohammad Yaser Abajy[1,2], Tuerkan Sakinc[4], Melanie Broszat[3,4], Katarzyna Schiwon[1], Guenther Koraimann[2], Walter Keller [2], and Elisabeth Grohmann[1,3,4]#

[1] Department of Environmental Microbiology/Genetics, University of Technology Berlin, 10587 Berlin, Germany

[2] Institute of Molecular Biosciences, University of Graz, 8010 Graz, Austria

[3] Faculty of Biology, Microbiology, Albert-Ludwigs-University Freiburg, 79104 Freiburg, Germany

[4] Division of Infectious Diseases, University Medical Centre Freiburg, 79106 Freiburg, Germany

Corresponding author. Mailing address: Division of Infectious Diseases, University Medical Centre Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany. Phone: +49 76127082520. Fax: +49 76127018200. E-mail: elisabeth.grohmann@uniklinik-freiburg.de

† Present address: Robert Koch-Institute, DGZ-Ring 1, D-13086 Berlin, Germany.

* KA and E-K C equally contributed to the work.

Running Title: Gram positive T4S protein TraG

Supplemental Methods

DNA preparation and transformation. Extraction and purification of plasmid DNA from *E. coli* was performed using QIAprep spin miniprep kit (Qiagen). Restriction endonucleases were purchased from Promega and New England Biolabs. T4 DNA ligase and shrimp alkaline phosphatase were from Roche Diagnostics. GenTherm DNA polymerase was purchased from Rapidozym (Berlin, Germany), *Phusion* high-fidelity DNA polymerase from Finnzymes, *Precissor* High-fidelity DNA polymerase from BioCat (Heidelberg, Germany) and Taq DNA polymerase was from Genscript (Scotch Plains, NJ). The enzymes were used as specified by the suppliers.

Synthetic oligonucleotides were purchased from Sigma-Aldrich. PCR fragments for cloning experiments were purified by Wizard SV gel and PCR Clean-Up system (Promega). Preparation of competent cells and *E. coli* transformations with plasmid DNA were performed by standard methods.

Cloning of *traG*ΔTMH and *traG* domains. *traG*ΔTMH (the first 45 amino acids of *traG* containing a putative transmembrane helix (TMH) were deleted), slt_{TraG} and $chap_{TraG}$ were amplified by PCR using the primers listed in Table S1. The respective EcoRI/SalI DNA fragments were inserted into EcoRI/SalI cut pMAL-c2X expression plasmid. The nucleotide sequences of the insertions were verified by sequencing at SMB (Rüdersdorf, Germany).

Site-directed mutagenesis of the SLT domain. Site-directed mutagenesis was performed by PCR overlap extensions (1). Briefly, two PCR products were generated with primer pairs *traG*_E87G-fw/*traG-Sal*I-rev and *traG-Eco*RI-fw/*traG*_E87G-rev thus exchanging the putative catalytic glutamate at TraG position 87 (Acc. No. CAD44387) for glycine, thereby introducing a BamHI site without further amino acid exchanges. The PCR products were

annealed and used as template in a PCR with primer pair *traG*Δ*tmh-Eco*RI-fw/*slt-Sal*I-rev. The PCR product was inserted into EcoRI/SalI cut pMAL-c2x, the nucleotide exchanges were verified by BamHI-restriction and sequencing.

Expression of fusion proteins. *E. coli* XL10 and *E. coli* BL21-CodonPlus(DE3)-RIL harboring the recombinant expression plasmids were grown in 200 ml LB broth supplemented with 100 µg/ml Ap and 50 µg/ml Cm at 37°C to OD_{600} of 0.6 (MBP-TraGΔTMH, 78.5 kDa; MBP-SLT_{TraG}, 57.3 kDa, MBP-$CHAP_{TraG}$, 64 kDa). For simplicity the MBP fusion proteins are referred to as TraGΔTMH, SLT_{TraG}, $SLT(E87G)_{TraG}$ and $CHAP_{TraG}$, respectively, in the text. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) and stopped after 3 hours due to the cell toxicity of the TraG protein. The *E. coli* cells were pelleted and re-suspended in 10 ml buffer A [100 mM K_2HPO_4/KH_2PO_4, 50 mM $(NH_4)_2SO_4$, 0.5 % Triton X-100 (pH 7.6)]. 1 mM PMSF, 1mM benzamidin and 10 µl protease inhibitor mix (Roche Diagnostics) were added.

Purification of MBP-tagged proteins. MBP-TraGΔTMH, MBP-SLT, MBP-SLT(E87G) and MBP-CHAP were purified in one step by affinity chromatography with 1 ml MBP Trap columns (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A containing 0.05 mM maltose and the proteins were eluted with buffer A containing 10 mM maltose. Protein fractions of high purity were pooled, dialyzed against buffer A to remove the maltose and concentrated by Amicon CentriPrep columns (molar mass cutoff < 30 kDa, Millipore) and applied to the biochemical assays. MBP used as negative control in the assays was purified following the same protocol. Rabbit polyclonal anti-TraG antibodies were produced by BioGenes (Berlin, Germany) with purified TraGΔTMH protein according to standardized protocols.

PG isolation and purification from *E. faecalis* according to Leclerc et al. (1989) (2) with some modifications: *E. faecalis* JH2-2 was grown in 1 l Todd-Hewitt broth (THB) supplemented with 25 µg/ml Rif to OD_{600} = 0.4. The cells were harvested at 7000 × g, 4°C for 15 min and washed with 80 ml 25 mM Tris/HCl buffer (pH 8.0). The pellet was vacuum-dried and suspended in 80 ml 4% SDS. The suspension was shaken at 150 rpm at room temperature for 90 min and sonicated (Branson Sonifier 250, Microtip, 40% output, level 8, 2 sec pulse, 8 sec pause) for 5 min. The extract was boiled for 15 min at 100°C and centrifuged at 12000 × g for 15 min. The pellet was re-suspended in 0.1% Triton X-100 (v/v) and incubated for 30 min at room temperature. The suspension was centrifuged as before, the pellet was washed four times with 80 ml distilled water, PG was lyophilized and stored at -80°C.

Furthermore, to exclude cross-contamination of the PG preparation with PG degrading enzymes bound to the PG, PG from *E. faecalis* JH2-2 was purified following the protocol of Eckert et al. (2006) (3) with some modifications: *E. faecalis* JH2-2 was grown in 2 l Brain Heart Infusion broth (BHI) (Oxoid) supplemented with 25 µg/ml Rif to OD_{650} = 0.7. The cells were harvested at 12 000 × g, 20°C for 10 min. PG was extracted by treating the bacterial pellet with 56 ml 4% SDS in a water-bath at 100°C for 30 min. The extract was centrifuged at 12 000 × g for 10 min at 20°C and washed 5 times with 80 ml distilled water. The pellet was re-suspended in 8 ml phosphate buffer (20 mM, pH 7.8) containing 200 µg/ml trypsin (Sigma-Aldrich) and digested overnight at 37°C with shaking at 150 rpm. Trypsin inhibitor (200 µg/ml, Sigma-Aldrich) in 8 ml phosphate buffer (20 mM, pH 7.8) was added and incubated at 37°C for 30 min with shaking at 150 rpm. The suspension was centrifuged as before, the pellet was washed three times with 80 ml distilled water, PG was lyophilized overnight and stored at -80°C. No differences regarding PG purity between the two methods were noticed.

PG isolation and purification from *E. coli* DH5α according to Rosenthal and Dziarski (1994) (4) with some modifications. 5 ml of an *E. coli* DH5α o/n culture were inoculated in

100 ml M9 minimal medium and incubated at 37ºC until the stationary phase was reached (OD_{600} of ca. 1.0). The cells were harvested, washed with 4 ml 10 mM Tris/Cl pH 6.8 and centrifuged again. The pellet was re-suspended in 3 ml 10 mM Tris/Cl pH 6.8, this suspension was added drop-wise to 30 ml of 4% boiling SDS and boiled for 45 min. The suspension was pelleted at 163,000 × g (50 Ti rotor, OTD combi ultracentrifuge, Thermo Fisher Scientific GmbH) for 20 min, the pellet was re-suspended in 15 ml 2 M NaCl and shaken o/n at room temperature. Another centrifugation step (163,000 × g, 20 min) followed, the pellet was washed with 6 ml distilled water, re-suspended in 500 µl distilled water and digested with the following enzymes: 50 µg/ml RNAse A (from bovine thyroid, Sigma-Aldrich), 50 µg/ml DNAse from *Bacillus* 9034-90-2 (Roche Diagnostics), 200 µg/ml α-amylase from *Bacillus* 9000-90-2 (Roche Diagnostics) and 200 µg/ml pronase E from *Streptomyces griseus* (Sigma-Aldrich) for 60 min at 60ºC. 166 µl 32% SDS were added and the suspension was boiled for 15 min, centrifuged as described above and the pellet was washed with 6 ml distilled water. This step was repeated three times. The final pellet was lyophilized and stored at -80°C.

Supplemental References

1. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
2. **Leclerc, D., and A. Asselin.** 1989. Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. Can. J. Microbiol. **35**:749–753.
3. **Eckert, C., M. Lecerf, L. Dubost, M. Arthur, and S. Mesnage.** 2006. Functional Analysis of AtlA, the Major N-Acetylglucosaminidase of *Enterococcus faecalis*. J. Bacteriol. **188**:8513–8519.
4. **Rosenthal, R., and R. Dziarski.** 1994. Isolation of peptidoglycan and soluble peptidoglycan fragments. Methods Enzymol. **235**:253–285.
5. **Eichenbaum, Z., M. Federle, D. Marra, W. de Vos, O. Kuipers, M. Kleerebezem, and J. Scott.** 1998. Use of the lactococcal *nisA* promoter to regulate gene expression in gram-positive bacteria: comparison of induction level and promoter strength. Appl. Environ. Microbiol. **64**:2763–2769.

Supplemental Tables

Table S1: Oligonucleotides used

Primer	Sequence (5´-3´)[a]	Position
***traG* in frame deletion**		
UPS_*traG*_XbaI-fw	CGC**TCTAGA**TTGCCTTACTCAACT	7141-7155[b]
UPS_*traG* _BamHI-re	ATA**GGATCC**AGCTGTTGCTCCCAT	43-57[c]
DWS_*traG* _BamHI-fw	CTC**GGATCC**GCAAGTTATGTTGA	1135-1148[c]
DWS_*traG* _EcoRI-rev	TAT**GAATTC**CCGTCTGATTCTGC	2160-2173[c]
Screening in frame deletion		
delta_*traG* _A-fw	AATGCGTTTGCTTAACCCAGG	6662-6682[b]
delta_*traG* _B-re	GATCCTGGACCACCTACTAC	2253-2272[c]
delta_*traG* _C-fw	TTCATATCATGGGAGCAACAGC	35-56[c]
delta_*traG* _D-re	TCCTTATTCAACATAACTTGC	1135-1155[c]
***aacA-aphD* gene**		
Gent_BglII-fw	TAT**AGATCT**GGACCTACATGATGAATGG	41998-42016[d]
Gent_BglII-rev	CTA**AGATCT**CTGGACTTGACTCACTTC	44018-44035[d]
Cloning of *traG*Δtmh and *traG* domains		
traGΔtmh-EcoRI-fw	TCC**GAATTC**CTAGCAACAGAA	178-189[c]
traG-SalI-rev	CTC**GTCGAC**AACTCCATTTCTTCCT	1157-1172[c]
slt-SalI-rev	CAC**GTCGAC**TTCTTAACCTGACATATT	556-573[c]
chap-EcoRI-fw	TGAGAATTCGGTGGCGAAAAGGTA	565-579[c]
PCR overlap extension		
traG _E87G-fw	GCAAATT**G*G*ATC*C***GGCGGTAATGGTG	294-319[c]
traG _E87G-rev	CGCC***G*GATC*C***AATTTGCATAAGGGCTA	284-310[c]
***traG* complementation**		
traG _SalI_fw	GCG**GTCGAC**ATGGGAGCAACAGCT	43-57[c]
traG _RBS_SalI_fw	GAG**GTCGAC**ATTAGGAAGGTTGGTG	17-32[c]
traG _SalI_rev	GCC**GTCGAC**TTATTCAACATAACTTG	1093-1110[c]
traGΔtmh-SalI-fw	TCC**GTCGAC**CTAGCAACAGAA	178-189[c]
traG-SalI-rev	CTC**GTCGAC**AACTCCATTTCTTCCT	1157-1172[c]

***traG*Δtmh sequencing**		
pEU327_Test_SalI_fw	CTTGCCAGTCACGTTACG	5
pEU327_Test_SalI_rev	GATCTGCGATATCCACTTC	5

[a] added restriction sites are shown in **bold** and exchanged nucleotides in *italics* and *underlined*.

[b] GenBank accession number L39769

[c] GenBank accession number AJ505823

[d] GenBank accession number AF051917

Supplemental Figure Legends

Fig. S1: Purification of TraGΔTMH, SLT_{TraG}, $SLT(E87G)_{TraG}$, and $CHAP_{TraG}$ as MBP fusion proteins via affinity chromatography. Samples were analyzed on a 12% SDS polyacrylamide gel. Samples from overexpression and the respective protein eluted from the amylose column are shown. Lane 1, molecular weight marker (Jena Bioscience, Jena, Germany): marker bands: 116 kDa, 97 kDa, 66 kDa, 38 kDa, and 28 kDa; lane 2, TraGΔTMH overexpression; lane 3, TraGΔTMH eluate (78 kDa); lane 4, SLT_{TraG} overexpression; lane 5, SLT_{TraG} eluate (57 kDa); lane 6, $SLT(E87G)_{TraG}$ overexpression; lane 7, $SLT(E87G)_{TraG}$ eluate (57 kDa); lane 8, $CHAP_{TraG}$ overexpression; lane 9, $CHAP_{TraG}$ eluate (64 kDa).